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Screening of potential lignin-degrading microorganisms and evaluating their optimal enzyme producing culture conditions

Master of Science Thesis [in Master of Biotechnology]

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Industrial Biotechnology

CHALMERS UNIVERSITY OF TECHNOLOGY

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Thesis for the Degree of Master of Science

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I. Abstract

Wood and other lignocellulosic biomass is a low cost and abundant resource that can be used in the large scale production of fuels and chemicals. The presence of covalent lignin-carbohydrate linkages between sugar hydroxyl of hemicelluloses and phenylpropane subunits in lignin gives lignocellulose protection against degradation. Separation and modification of the lignocellulosic materials are required in order for it be utilized, where microbial modification of the biomass is one alternative. The aim of this project is to identify strains of filamentous fungi with high potential in the degradation of lignocellulose compounds. Birch wood and wheat bran were used as substrate for the induction of the production of lignocelluloses degrading enzymes in fungi. Seven strains of filamentous fungi were chosen and they were screened for their ability to grow on birch containing agar medium and decolorization of synthetic dye, RBBR, methyl green and guaiacol as an indicator for the production of lignin degrading enzymes. Among seven strains of fungi, *Penicillium pinophilum* (IBT 10872) and *Trametes hirsuta* (NCIM 1201) were chosen for cultivation in different liquid media and solid state culture (SSC) and the amount of enzyme produced were analysed. Both *P. pinophilum* and *T. hirsuta* were found to produce higher amount of cellulase and xylanase enzymes when they were grown in birch wood liquid medium containing peptone compare to medium without peptone. Moreover, solid state culture of *T. hirsuta* on wheat bran was also found to induce the lignin degrading enzyme, laccase, but no manganese peroxidase activity was detected.

II. Acknowledgement

First of all, I would like to express my sincere gratitude to my examiner, Professor Lisbeth Olsson for her guidance and supervisions throughout this thesis work. I am also grateful to my supervisor, George Anasontzis for dedicating his precious time, giving advice and helping me from the beginning to end of this thesis. This work would not succeed without their great supports. I also like to thank SEKAB E-Technology, Örnskoldsvik, Sweden for supplying birch wood, the main substrate used in this project. I would also like to extend my thanks to all the members in the Department of Chemical and Biological Engineering, Industrial Biotechnology and System Biology groups for lending a hand to assist me in my laboratory work. Last but not least, a special thanks to my parents and family members for encouraging me with love and care.

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1. Introduction

Lignocellulose describes the three major constituents in plants, namely cellulose, hemicelluloses and lignin. The composition of lignocelluloses depends not only on the species but also on the growth conditions, the different parts of the plant and their age (Jørgensen 2003). The abundance of lignocellulosic materials make them potentially inexpensive and readily available natural resources for the manufacturing of high value compounds and for biofuels production in a sustainable environment. The use of these materials involves a separation of the polymeric compounds - cellulose and hemicelluloses. The spontaneous degradation of these compounds are extremely slow, but there are microorganisms in soil and in the rumen of ruminants, capable of degrading them to sugars which then can be utilized as energy and carbon source by various microorganisms for the production of different products. These organisms are capable of growing on lignocellulosic materials and thus produce a wide range of enzymes that could be of scientific or industrial importance. The aim of this master thesis is to identify strains of microorganisms with high potential in the degradation of lignocellulose materials. Filamentous fungi were first screened for their ability to grow on wood containing medium using birch wood as growth substrate and their dye decolorization ability as an indicator for expression of ligninolytic enzymes. Two microorganisms were chosen for further study by growing them on birch wood or wheat brain containing liquid media and solid state culture to identify the effect of different substrate on growth and the induction of enzyme production. The cellulose and hemicelluloses degrading enzymes, cellulases and xylanases respectively, were measured to follow the growth of the organisms. The enzymes for lignin degradation, laccase and manganese peroxidases production levels were also determined.

2. Literature Review

This literature review contains information on the three main components of wood, their chemical composition and degradation, strains of filamentous fungi and lignocellulose substrates that will be used in this project.

2.1 Chemical composition and degradation of lignocelluloses

Wood consists of about 45% of cellulose, 20 – 30 % hemicelluloses and 20 - 40 % lignin depending on the wood species, their parts and age. (Schmidt 2006).

2.1.1 Cellulose

Cellulose, the major chemical component of the fiber wall, is a homopolysaccharide composed entirely of D-glucose linked together by β-1,4-glycosidic bonds with degree of polymerization ranging from 1,000 in bleached kraft pulps to 10,000 in native wood (Schmidt 2006). Each glucose unit is rotated 180^{o} relative to the adjacent one and the smallest repetitive unit of cellulose is cellobiose, two glucose units. Cellulose is a linear structure that has a strong tendency to form intra- or intermolecular hydrogen bonds resulting in the formation of cellulose microfibrils which promote aggregation into crystalline, high order regions. The arrangement of cellulose and microfibrils in plant cell wall is shown in Figure 1. The regions within microfibrils with less order are termed amorphous. The arrangement of crystalline and amorphous cellulose results in interesting properties of stiffness and rigidity on one hand and flexibility on the other hand. Moreover, the structure of cellulose with its hydrogen bond makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation (Jørgensen 2003). The enzymes for cellulose degradation belong predominantly to hydrolases, which cleave the glycosidic bonds. Cellulose is hydrolyzed by cellulase (endoglucanase), 1,4-β-cellobiosidase and β-glucosidase (Schmidt 2006).

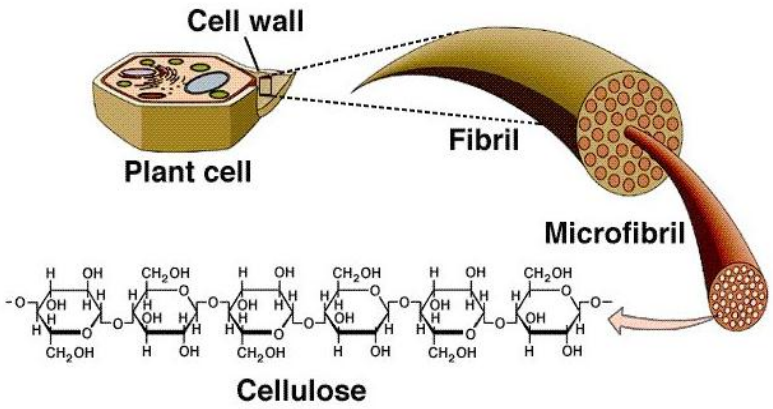

Figure 1: Arrangement of fibrils, microfibrils and cellulose in plant cell wall

The enzymes for the degradation of celluloses and hemicelluloses belong predominantly to the hydrolases which cleave glycosidic bonds by hydrolysis. A cellulases system which hydrolyses cellulose consists of cellobiohydrolases (exoglucanases, EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). The cellobiohydrolase and endoglucanase work synergistically by hydrolyzing 1,4-β-D-glycosidic linkages in cellulose, cello-oligomers and other β-D-glucans releasing cellobiose from the non-reducing ends, which are then degraded by β-glucosidase to glucose units as shown in Figure 2 (Schmidt 2006). Different microorganisms can produce variant of each enzyme group. The presence of the different components of the cellulose enzymatic system in an appropriate ratio is important in biorefineries to avoid, e.g., the repression of cellobiohydrolases by the accumulation of cellobiose due to a low activity of β-glucosidases (Jørgensen et al. 2006).

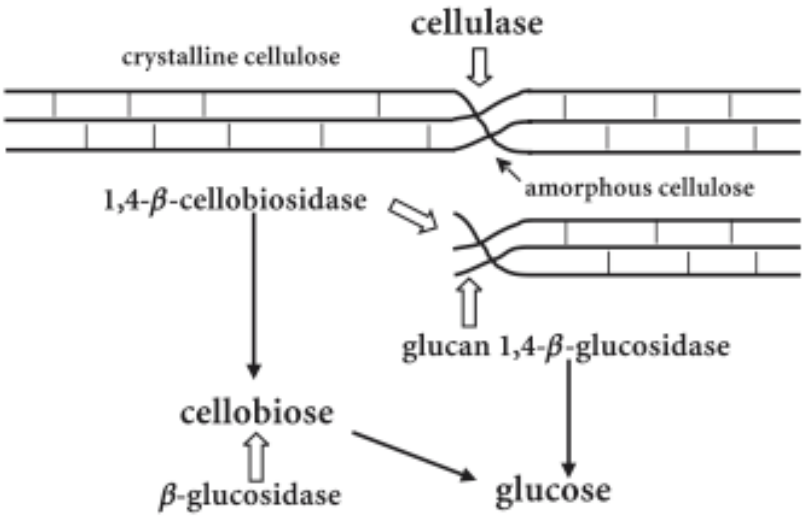

Figure 2: Diagram of enzymatic cellulose degradation (Schmidt 2006)

2.1.2 Hemicellulose

Hemicelluloses are complex heterogeneous polysaccharides made up of different monomeric residues, such as D-xylose, D-glucose, D-arabinose, D-mannose and D-glucuronic acid. Hemicelluloses have a lower degree of polymerization, (DP 50 – 300) compared to cellulose, they have side chains that can be acetylated and are essentially amorphous. They are classified according to the monomeric sugar in the backbone of the polymer, e.g. mannan (β-1,4-linked mannose) or xylan (β-1,4-linked xylose) hemicelluloses. The main chain of glucose and mannose residues are usually connected with β-(1,4) bond while the side chain is attached to main chain via α-(1,6) bonds in galactoglucomannan as shown in Figure 3. Xylan can be degraded by endo-1,4-β-xylanase (EC 3.2.1.8) and 1,4-β-xylosidase (EC 3.2.1.37) to xylose (Jørgensen 2003).

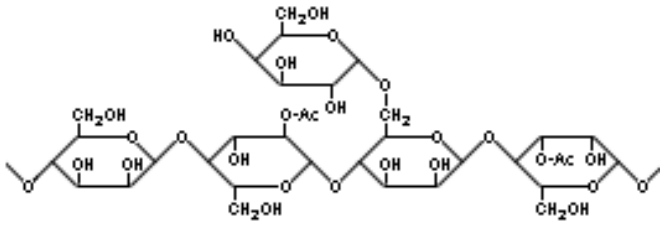

Figure 3: Schematic structure of O-acetyl-galactoglucomannan

Due to the more heterogenous nature of hemicellulose compared to cellulose, a complex mixture of enzymes is required for its degradation, such as endoxylanases, β-xylosidases, endomannanases, β-mannosidases, α-L-arabinofuranosidases and α-galactosidases (Jørgensen H. et al. 2005). The major hemicellulose of hardwoods is xylan, whose contents vary from 15 to 35%, while birch wood contains 22-30% xylan. The enzymatic xylan degradation is shown in Figure 4. The xylan backbone is hydrolyzed by the endo-1,4-β-xylanase to oligomers, xylobiose and xylose. Oligosaccharides are then degraded by xylan 1,4-β-xylosidase into xylose units from non-reducing ends. The side groups, acetyl group and 4-O-methylglucuronic acid that are linked to xylan backbone are also cleaved by acetylesterase (acetic-ester acetylhydrolase, EC 3.1.1.6) and xylan α-D-1,2-(4-*O*-methyl) glucuronohydrolase (EC 3.2.1.131) respectively. The degradation of hemicellulose is more common in wood fungi than in bacteria (Schmidt 2006).

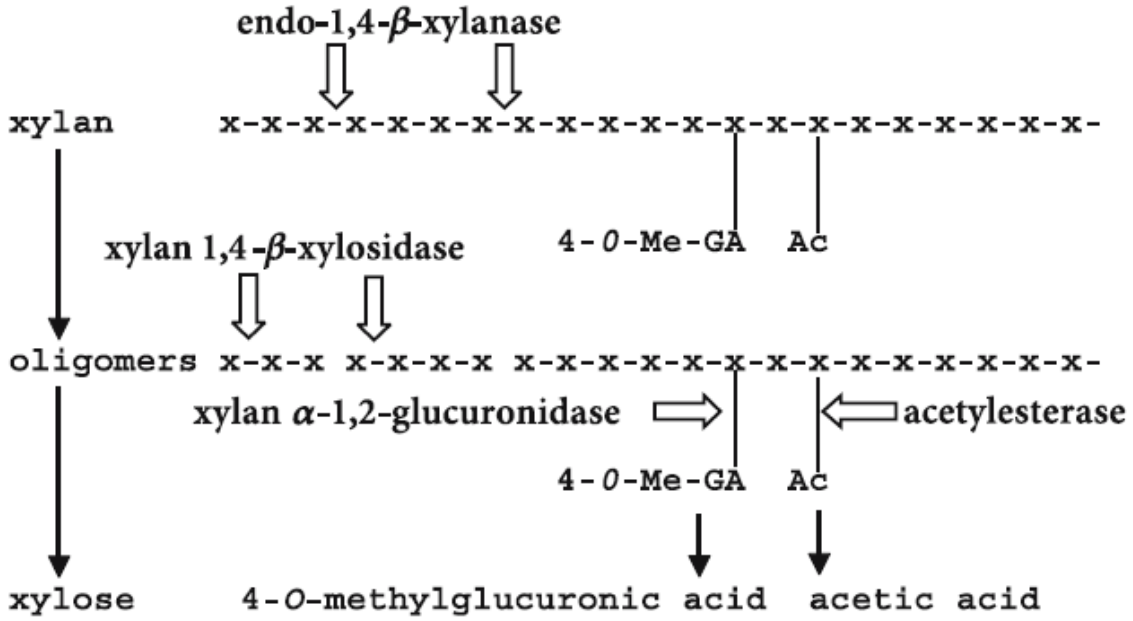

Figure 4: Diagram of enzymatic xylan degradation. x xylose residue, Ac acetic acid residue, 4-O-Me-GA 4-O-methylglucuronic acid residue, ⇑ degradation (Schmidt 2006)

2.1.3 Lignin

The third main constituent in wood, lignin, is a complex macromolecule formed by the dehydrogenative polymerization of three phenyl propane units (shown in Figure 5) namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol joined through ether bonds. The structure of lignin varies widely within species. It provides compressional strength to the cell wall of plant while cellulose provides the plant with flexible strength. In contrary to cellulose, lignin is a complex, three-dimensional macromolecule and is highly hydrophobic. It forms an amorphous complex with hemicelluloses enclosing cellulose and thus prevents the microbial degradation of accessible carbohydrates within wood cell wall. Moreover, its aromatic rings make it more difficult to degrade (Schmidt 2006). Lignin also acts as a cementing component to connect cells and harden the cell wall of xylem which helps the smooth transportation of water from roots to leaves. Ester linkages occur between the free carboxy group in hemicellulose and the benzyl groups in lignin, the lignin-carbohydrate complex (LCC), embeds the cellulose thus giving protection against microbial and chemical degradation (Jeffries 1994). Cellulose and hemicellulose are rather easily degradable, while lignin is

resistant to degradation by most microorganisms due to its phenylpropane units in the structure and the recalcitrant linkages between them (Schmidt 2006). Thus, there is great interest in finding organisms capable of breaking down lignin and in the cleaving of the chemical bonds that exist between lignin and hemicelluloses (LCC). These organisms might be producing enzymes that could be used in the modification of plant biomass.

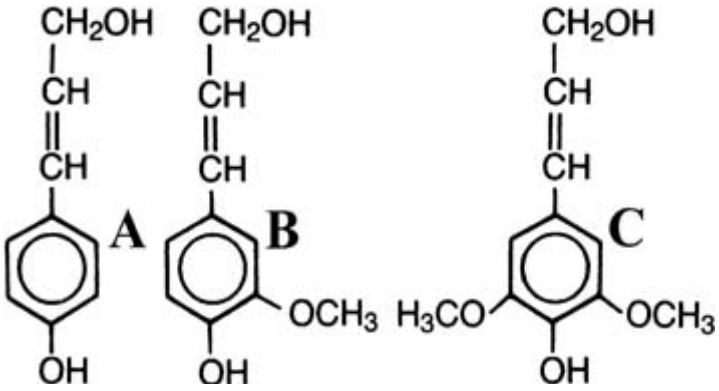

Figure 5: Building unit of lignin. (A) p-coumaryl alcohol, (B) coniferyl alcohol, (C) sinapyl alcohol (Schmidt 2006)

Lignin can be effectively broken down by a group of filamentous fungi known as white-rot fungi, which are capable of producing a number of oxidoreductases, enzymes able to attack phenolic structures. Lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase are among them (Hofrichter 2002).

Lignin peroxidase, LiP, EC 1.11.1.14 is a glycoprotein that contains heme, cleaves C-C bonds and oxidizes benzyl alcohols to aldehydes or ketones. LiP attacks both phenolic and non-phenolic lignin substructures by a one-electron oxidation reaction to generate unstable aryl radical cations. It requires an extracellular hydrogen peroxide, H_2O_2, as an electron acceptor. LiP initiates different non-enzymatic reactions such as the cleavage of C_α-C_β bond in the side chain, β-O-4 bond between side chain and next ring and cleavage of aromatic ring as shown in Figure 6 (Schmidt 2006).

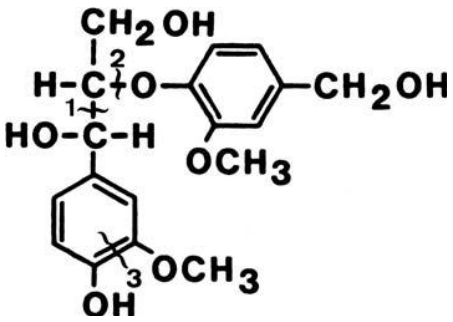

Figure 6: Reactions initiated by lignin peroxidase, (1) cleavage of Cα-Cβ bond in the side chain, (2) β-O-4 bond between side chain and next ring and (3) cleavage of aromatic ring (Schmidt 2006)

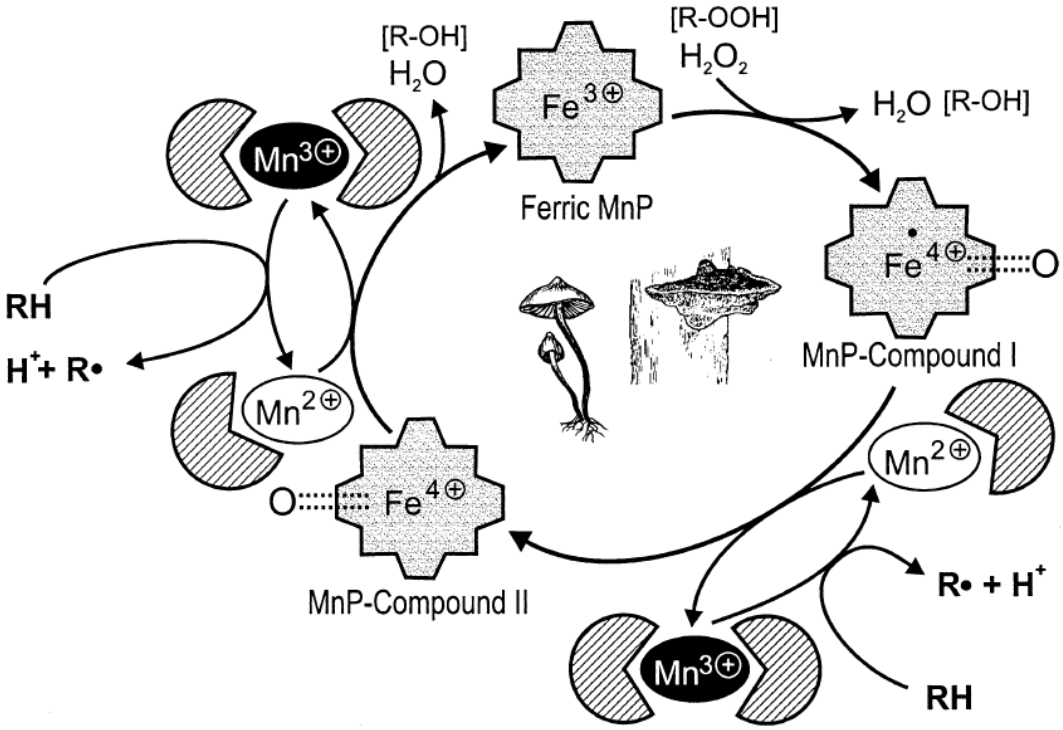

Figure 7: Catalytic cycle initiated by Manganese Peroxidase (Hofrichter 2002)

Manganese peroxidase, MnP, EC 1.11.1.13, is a heme containing glycoprotein, involved in lignin degradation by mainly attacking phenolic lignin component. The production of MnP is limited to certain basidiomycetes and some wood decaying white-rot fungi, which secrete MnP in multiple forms into their environment. It oxidizes phenolic structures by oxidizing Mn^{2+} to Mn^{3+} in the presence of H_2O_2. The catalytic cycle of MnP is initiated by binding of H_2O_2 to the native ferric enzyme and the formation of iron-peroxide complex (Figure 7). The

cleavage of oxygen-oxygen bond of hydrogen peroxide or other peroxide to one water molecule requires two electron transfers from the heme, resulting in the formation of MnP Compound I (Fe^{4+}-oxo-porphyrin-radical complex). MnP Compound I is then reduced to MnP Compound II (Fe^{4+}-oxo-porphyrin complex) by a monochelated Mn^{2+}, which is oxidized to Mn^{3+}. The native enzyme is then regenerated from the further reduction of MnP Compound II by Mn^{2+} and the releasing of another water molecule (Hofrichter 2002). The formed Mn^{3+}, a strong oxidant, then oxidizes phenolic structures by single electron oxidation (Schmidt 2006).

The third enzyme involved in lignin degradation is laccase, EC 1.10.3.2, in the family of copper-containing polyphenol oxidases, s also called a blue multicopper oxidase (Arora and Sharma 2010). It catalyzes the oxidation of a wide range of substrates such as mono-, di- and poly-phenols, aromatic amines and non-phenolic compound to free radicals and the four-electron reduction of oxygen directly to water. Then, the formed free radicals undergo numerous spontaneous reactions which in turn result in various bond cleavages of aromatic rings and other bonds (Leonowicz et al. 2001). However, laccase can't directly oxidize all potential substrates either due to the large size of the substrates which restrict their penetration into the enzyme active site or due to their high redox potential. Thus, it is required to have suitable chemical mediators that act as an intermediate substrate for laccase, whose oxidized radical formed are then able to react with the high redox potential substrates (Arora and Sharma 2010).

2.2 Lignocelluloses degrading organisms

A wide range of microorganisms including bacteria and fungi are capable of producing cellulases and hemicellulases but only a limited number of these microorganisms are capable of producing lignin degrading enzymes. Among these microorganisms, filamentous fungi appear to be the most efficient producers of enzymes degrading lignocellulose (Jørgensen 2003). The ability to produce specific enzymes for degradation of different carbon and nitrogen sources are due to the diverse habitat they are found (Prathumpai 2003).

White rot fungi, which mostly belong to the Basidiomycota phylum, have the ability to degrade lignin completely to carbon dioxide and water. They secrete three classes of extracellular ligninolytic enzymes: a phenol oxidase (laccase), two heme-containing

peroxidases (lignin peroxidase, LiP, and manganese peroxidase, MnP) and oxidases that produce H_2O_2 needed for peroxidase activities. LiP is capable of oxidizing phenolic and non-phenolics lignin substructures directly while most of laccases and MnP are only capable of oxidizing phenolic substructures (Koshijima et al. 2003).

In this project, seven strains of filamentous fungi belonging to Basidiomycota and Ascomycota phyla are chosen to study their ability to produce lignocellulose degrading enzymes. The first microorganism, *Trametes hirsuta* is a plant pathogen, found on dead wood of deciduous tree and it features a white and gray cap surface. It is a white rot fungus that belongs to the Basidiomycota phylum and has been found to produce a wide range of enzymes, such as cellulases, cellobiose dehydrogenase, laccase, manganese peroxidase (Nakagame 2006, Nerud 1991, Rodríguez 2005). It has been found to produce 207 U/L and 2000 U/L of laccase when grown on orange peel in solid state fermentation and expanded-bed-reactor, respectively (Böhmer et al. 2011 and Couto et al. 2006). Two Penicillium species namely *P. pinophilum* and *P. thomii* are capable of producing xylanases and cellulases (Jørgensen 2006). *P. thomii* has been found to produce 44 U/ml of endo-xylanase on minimal salt medium while *P. pinophilum* has been found to produce 104 U/ml of xylanase when grown on birch wood xylan (Palaniswamy et al. 2008 and Li et al. 2006). Different strains of *P. pinophilum* produced different amount of enzymes depending on the substrate they were grown on (Data shown in Table 1). The forth strain of fungi, *Phoma herbarum*, is a plant pathogen hosted on *Picea sp*. and *Pinus sp*. and it can also grow on dead wood (Farr and Rossman, Fungal databases). Laccase activity can be found in some of the *Phoma* sp. The fifth strain is *Davidiella tassiana*, also known as *Cladosporium herbarum*, belongs to the phylum Ascomycota (Farr and Rossman, Fungal databases). It is also a plant pathogen found on eucalyptus, *Citrus sp., Picea sp., Pinus and Populus sp.* The last two strains of fungi, which belong to Basidiomycota, are *Ustilago maydis* found to produce laccase and *Sphaerulina polyspora*. *U. maydis* is a plant pathogenic fungus that induces tumors on host plant and it is also the fungus that causes corn smut disease. When grown in synthetic media, it grows in yeast-like colonies and it is an important model system used in studying of pathogen-host interactions (BOARD Institute of MIT and Harvard*).

*(http://www.broadinstitute.org/annotation/genome/ustilago_maydis.2/Info.html)

Table 1: Lignocellulose-degrading enzyme activities of different fungal strains on lignocellulose substrates

Strains	Substrate and cultivation	Enzyme	Amount produced	Reference
T. hirsuta (BT2566)	Orange peel, expanded-bed reactor	Laccase	2000 U/L	Couto et al. 2006
T. hirsuta (BT2566)	Orange peel, Solid state fermentation	Laccase	207 U/L	Böhmer et al. 2011
P. thomii	Minimal salt medium, Fermentation	Endo-xylanase	44 U/ml	Palaniswamy et al. 2008
P. pinophilum (NTG 1II/6)	Avicel + barley straw, submerged fermentation	Xylanase	27 U/ml	Li et al. 2006
P. pinophilum	Birch wood xylan, shake flask fermentation	Xylanase	104 U/ml	Lee et al. 2011
P. pinophilum (TERI DB1)	Corn cobs molasses, Solid state fermentation	Laccase	0.3 U/ml	Pant and Adholeya 2007
		MnP	3.3 U/ml	
	Wheat straw with effluent, submerged fermentation	LiP	33U/ml	

2.3 Lignocellulosic substrates

Two types of substrates are used in this study as a carbon source for the growth of the microorganisms: birch wood and wheat bran. Birch is a hardwood tree that belongs to the genus *Betula* sp. and is mainly used in pulp, paper industry and in furniture-making. The chemical composition of birchwood differs from species to species. In general, it is composed of approximately 14% non-cell wall materials, 42.7% cellulose, 26.4% hemicellulose and

16.9% lignin (Thomsen et al. 1999). Wheat is a grass belonging to *Triticum* sp. which is cultivated worldwide mainly as a food crop. It contains higher protein content than most of the cereals such as maize and rice. Wheat bran is the hard outer layer of the wheat grain and it is particularly rich in essential fatty acids and dietary fibers and contains significant amount of proteins, starch, vitamin and minerals. Differences in the production of ligninolytic enzymes were expected by using different lignocellulosic substrates which may contain significant concentrations of soluble carbohydrates and inducer of enzyme expression.

3. Materials and Methods

3.1 Microorganisms

Microorganisms used in this study, listed in Table 2, were obtained from the Industrial Biotechnology Filamentous Fungi Strains Database (Chalmers University of Technology, Sweden). These strains were re-cultured and maintained on Potato Dextrose Agar (PDA) at 4^{o}C.

Table 2: Strains information of filamentous fungi used

Strains	**Indbio_ID**	**NCIM or other number**	**Description**
Penicillium thomii	PTH IB.0001	-	Isolated strain obtained from KTH, Wallenberg Wood Science Center (WWSC) lignocellulose project
Penicillium pinophilum	PPI IB.0001	IBT 10872	Strain obtained from the DTU fungal strain collection
Phoma herbarum	PHE IB.0001	-	Isolated strain obtained from KTH, WWSC lignocellulose project
Sphaerulina polyspora	SPO IB.0001	-	-
Davidiella tassiana	DTA IB.0001	-	Isolated strain obtained from KTH, WWSC lignocellulose project
Ustilago maydis	UMA IB.0001	NCIM 983	Strain obtained from the National Collection of Industrial Microorganisms (NCIM) in India
Trametes hirsuta	THI IB.0001	NCIM 1201	Strain obtained from the National Collection of Industrial Microorganisms (NCIM) in India

3.2 Media and culture conditions

Potato Dextrose Agar (PDA) and minimal media containing 5% v/v stock salt solution, 0.02 % v/v Hutner's trace elements, 1 % w/v glucose and 1.5 % w/v agar were used to maintain the microorganisms. Stock salt solution contained the following (per liter): $NaNO_3$ 6 g, KCl 0.52 g, $MgSO_4.7H_2O$ 0.52 g and KH_2PO_4 0.82 g. Hutner's trace elements solution consisted of (per liter) $ZnSO_4.7H_2O$ 2.2 g ,H_3BO_3 1.1g , $MnCl_2.4H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.5 g, $CoCl_2.6H_2O$ 0.16 g, $CuSO_4.6H_2O$ 0.16 g, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.11 g and Na_2EDTA 5 g.

Three different sets of experiments were performed. The first part was the screening for the ability of the selected fungi to grow on wood-containing agar media and their ability in synthetic dye decolorization as an indicator for the expression of lignin degrading enzymes. The aim of the second part was to determine the potential expression of lignocellulose degrading enzymes by the organisms in liquid media and the third part was the enzymes production in solid state cultures (SSC).

Screening tests for the ability of organisms to grow on wood-containing agar plates were performed in birch wood agar medium with and without addition of glucose in the medium. The medium contained 2 % w/v birch wood powder (1mm Hammermilled) obtained from SEKAB E-Technology, Örnskoldsvik, Sweden, with or without 0.05 % w/v glucose, 5 % v/v stock salt solution, 0.02 % v/v Hutner's trace element and 1.5 % w/v agar. In dye decolorization tests, PDA medium containing 0.04 % w/v Remazol Brilliant Blue R, RBBR, (R8001, Sigma), 0.04 % w/v methyl green (M5015, Sigma) or 0.01% w/v guaiacol (G5502, Sigma) were used. Guaiacol was added into PDA before sterilization while RBBR and methyl green were added as a sterilized filtered solution. Each fungal strain was inoculated onto all media, incubated at 30^{o}C and the growth was followed for a period of 2 weeks. The cultivation was done in duplicate.

Lignocellulose degrading enzymes production was assessed on 3 different liquid media.

1. Birch wood liquid medium containing 2 % (w/v) birch wood powder, 0.05 % (w/v) glucose, 5 % (v/v) stock salt solution and 0.02 % (v/v) Hutner's trace elements. [*T. hirsuta* (Tb1) and *P. pinophilum* (Pb1)]

2. Birch wood liquid medium containing 2 % (w/v) birch wood powder, 0.06 % (w/v) peptone, 0.05 % (w/v) glucose, 5 % (v/v) stock salt solution and 0.02 % (v/v) Hutner's trace elements. [*T. hirsuta* (Tb2) and *P. pinophilum* (Pb2)]

3. Wheat bran liquid media containing 2% (w/v) wheat bran (Vetekli 40% kostfibrer, Kungsörnen, Lantmänen, Sweden), 0.05 % (w/v) glucose, 5 % (v/v) stock salt solution and 0.02 % (v/v) Hutner's trace elements. [only *T. hirsuta* (Tw)]

The Erlenmeyer flasks filled with 150 ml of liquid media were autoclaved at 121^{o}C for 20 min. Mycelia from an agar plate were harvested and used for inoculation. Cultures were done in triplicate for every microorganism. The flasks were incubated at 100-120 rpm, at 30^{o}C and samples were collected regularly for 2 weeks. The collected samples were centrifuged at 5100 rpm for 15 minutes and the supernatant was stored at -20^{o}C for further analyses.

Enzyme production on solid state culture was performed by cultivating *T. hirsuta* on birch wood (SSCTb) and wheat bran. *T. hirsuta* (SSCTw) was cultivated in 250 ml Erlenmeyer flasks containing 5 g dry weight birch wood powder and in 100 ml Erlenmeyer flasks containing 5 g dry weight wheat bran. After sterilizing the substrate at 121^{o}C for 20 minutes, 2 ml stock salt solution was added and the moisture content was adjusted to 90 % relative humidity with sterilized distilled water. The cultures were inoculated aseptically by adding 15 ml of distilled water into each of 3 agar plates containing fungi which was then mixed and vortex well before transferring 1 ml into each solid state medium. All the flasks were incubated at 30^{o}C and the moisture content was maintained throughout the cultivation period.

3.3 Enzyme extraction

Crude extract of SSC was obtained by adding 50 ml and 75 ml of sterilized distilled water to the contents of each wheat bran flask and birch wood flask respectively, and stirred for 1 hr at 4^{o}C follow by centrifugation. The supernatant was stored at -20^{o}C until further analysis.

3.4 Enzyme assays and protein determination

Xylanase activitiy was determined by measuring the release of reducing sugars from xylan with the DNS method (Souza et al. 2006). The reaction mixture contained 20 µl of culture supernatant or SSC extract and 180 µl of 2 % w/v birch wood xylan in 0.1 M phosphate buffer, pH 6.5 or 0.1M sodium acetate buffer, pH 5 for *T. hirsuta* and *P. pinophilum* respectively. Xylose was used as standard. One unit of enzyme activity (U) is defined as the amount of enzyme that released 1µmole xylose equivalent in 1 minute, expressed as U/L or µmole/Lmin. Cellulase activities were measured using 2 % w/v carboxymethyl cellulose in 0.1 M phosphate buffer, pH 6.5 as substrate for *T. hirsuta* enzyme and in 0.1M sodium acetate buffer, pH 5 for supernatant from *P. pinophilum* and glucose as standard. Absorbance at 540 nm was measured using Thermo Scientific GENESYS 20 Visible spectrophotometer. One unit of enzyme activity (U) is defined as the amount of enzyme that released 1µmole glucose equivalent in 1 minute. Heat inactivated samples at 100^{o}C for 15 minutes were included as controls. All assays were performed at 30^{o}C in triplicate.

Laccase activity was determined by monitoring the oxidation of ABTS (ε = 29,300 M^{-1} cm^{-1}) (2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid) in 100 mM sodium acetate buffer, pH 5. The reaction mixture contained 10 mM ABTS in 100 mM sodium acetate buffer and enzyme supernatant in a total volume of 1 ml. The oxidation of ABTS was measured by an increase in absorbance at 436 nm (Mansur et al. 2003). One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 µmole per minute of oxidized product.

Manganese peroxidase activity was measured based on the oxidation of Mn^{2+} to Mn^{3+} and the formation of Mn^{3+}- malonate complex from 1 mM $MnSO_4$ (ε = 11,590 M^{-1} cm^{-1}) in 0.05 M disodium malonate, pH 4.5. The reaction mixture (1 ml) contained 0.05 M disodium malonate (pH 4.5), 1 mM $MnSO_4$, 0.1 mM H_2O_2 and the medium supernatant. The reaction was initiated with the addition of H_2O_2 and the rate of product formation was monitored at 270 nm (Fujihara et al. 2010). The laccase and MnP activity were measured using BECKMAN COULTER- DU® 800 UV/Visible Spectrophotometer.

The laccase and MnP activity were calculated from the following formula derived from Beer-Lambert Law

$$c = \frac{10^6 \Delta A}{\varepsilon \Delta t}$$

Where c = Enzyme activity (U/L)

ΔA = Increase in absorbance at 436 nm

ε = Molar absorption coefficient

Δt = Reaction time in minute

$\Delta A / \Delta t$ = slope from graph

The concentration of extracellular protein was analyzed by using Bio-rad total protein assay based on Bradford method with bovine serum albumin (BSA) as standard. The absorbance at 595 nm was measured with a BMG LABTECH- FLUOstar Omega, multi-mode microplate reader.

4. Results

4.1 Screening test

Seven strains of filamentous fungi were first screened for the ability to grow on birch wood containing solid medium and the ability to decolorize the synthetic dye. Based on the results of the screening tests, two microorganisms were selected for further studies.

4.1.1 Birch wood as substrate

Table 3: Growth of filamentous fungi on birch wood agar medium

Fungal Strains	Birch wood + glucose	Birch wood
Trametes hirsuta	++	+
Penicillium pinophilium	++	++
Sphaerulina polyspora	++	+
Penicillium thomii	+	+
Ustilago maydis	++	-
Davidiella tassiana	+	+
Phoma herbarum	-	-

Note: - = no growth

+ = total growth of less than 2.5 cm in size

++ = total growth of more than 2.5 cm in size

The growth morphology of the fungi grown on PDA was shown in Appendix I. All strains were screened for their ability to grow on birch wood with or without addition of glucose, as an indicator of the production of lignocellulose degrading enzymes. Glucose was added as an inducer for growth at the beginning of cultivation. Table 3 shows the results for the growth of fungi after one week of incubation at 30^{o}C. The growth was monitored visually and each sign was given based on the total size of all the colonies seen on the agar plate. Most of the strains grew well on birch wood, supplemented with glucose. However, all examined strains, except from *P. herbarum* and *U. maydis* were able to grow adequately even in the absence of supplemented glucose, indicating the capability to produce enzymes necessary for the degradation of the wood polymers.

4.1.2 Indicator dyes as substrate

Table 4: Dye decolorization effect during growth of different fungal strains on chromophore indicators

Fungal strains	RBBR	Methyl green	Guaiacol
T. hirsuta	+	No growth	+
P. pinophilum	+	No growth	+
S. polyspora	+	+	-
P. thomii	+	+	-
U. maydis	+	-	-
D. tassiana	-	-	-

Note: + = Positive for decolorization of dye

- = Negative for decolorization of dye

The ability of the strains to decolourize the three different indicators, RBBR, methyl green and guaiacol, were examined on PDA plates with 0.04 % w/v, 0.04 % w/v and 0.01 % w/v of the respective indicator. The results are shown in Table 4. The positive reactions with RBBR and methyl green are observed as a colorless halo around the microbial growth while with

guaiacol, a positive reaction is indicated by the formation of a reddish-brown halo. Five out of six strains examined were positive on RBBR indicator plate except for *D. tassiana* which was negative in all three plates. The positive results indicated the production of lignin-degrading enzymes which decolourize the polymeric dyes.

Due to their capability to grow on birch wood and the positive decolourization results, *T. hirsuta* and *P. pinophilum* were selected for further investigation of their lignocellulose degrading enzymatic system. Moreover, *T. hirsuta* exhibited fast growth rate on the screening media, as full growth on the plate dish was observed even after 24 hours of cultivation, contrary to the other positive strains, for which it took more than 48 hours before the first colony was observed.

4.2 Cultivation of Penicillium pinophilum on liquid medium

Figure 8 below shows the different morphology of *P. pinophilum* on different agar plate. The color of the mycelia was green on minimal medium while it was more yellowish on PDA.

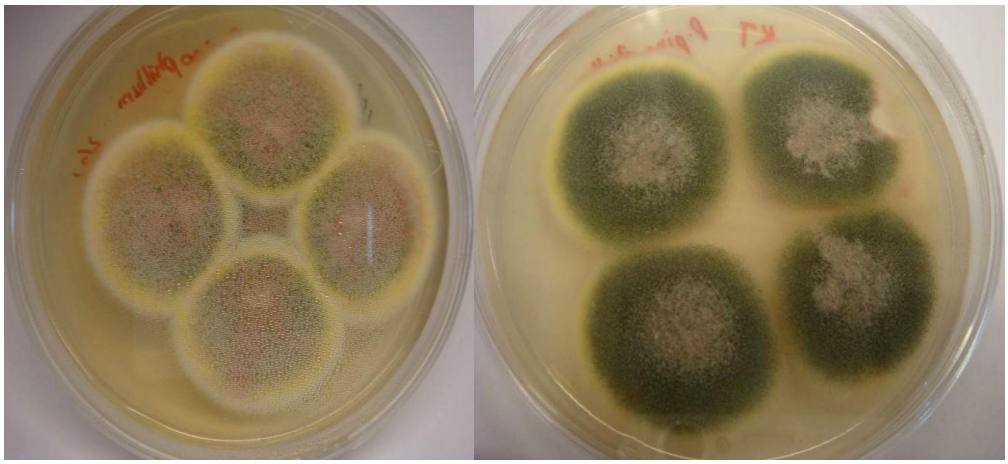

Figure 8: Morphology of the colonies of *P. pinophilum* on PDA (left) and minimal medium (right)

Shake flask cultivation of *P. pinophilum* was performed in triplicate in birch wood liquid medium without peptone (Pb1) and in duplicate in birch wood liquid medium containing peptone (Pb2). The production of cellulose and hemicellulose degrading enzymes, cellulases

and xylanases respectively, was initially assayed at pH 6.5, exhibiting low respective enzymes' activity. It was observed, however, that with an initial pH of 5.7, *T. hirsuta* was altering it towards neutral values and *P. pinophilum* to pH 5 (Data shown in Appendix II). The change in the pH of the growth medium might be due to the production of extracellular metabolites that acidify (organic acids production such as oxalic acid via citric acid cycle) or alkalize (consumption of anions or the formation of ammonia from proteins and amino acids) the medium. The pH value influences generation of spores, mycelial growth and enzyme activity (Schmidt 2006). Thus it is possible to assume that the enzymes they produce would be more active at the respective pH condition. Thus, the enzyme activity assays were performed at pH value closer to the culture medium. In order to test whether the pH of assay condition affect the enzyme activity, the cellulase assay of Pb2 were performed at pH 5 with 0.1 M sodium acetate buffer and at pH 6.5 with 0.1 M phosphate buffer. It can clearly be seen in Figure 9 that cellulase activities are higher at pH 5 compared to the activities observed at pH 6.5. Thus, the entire cellulase and xylanase assay for *P. pinophilum* were performed at pH 5.

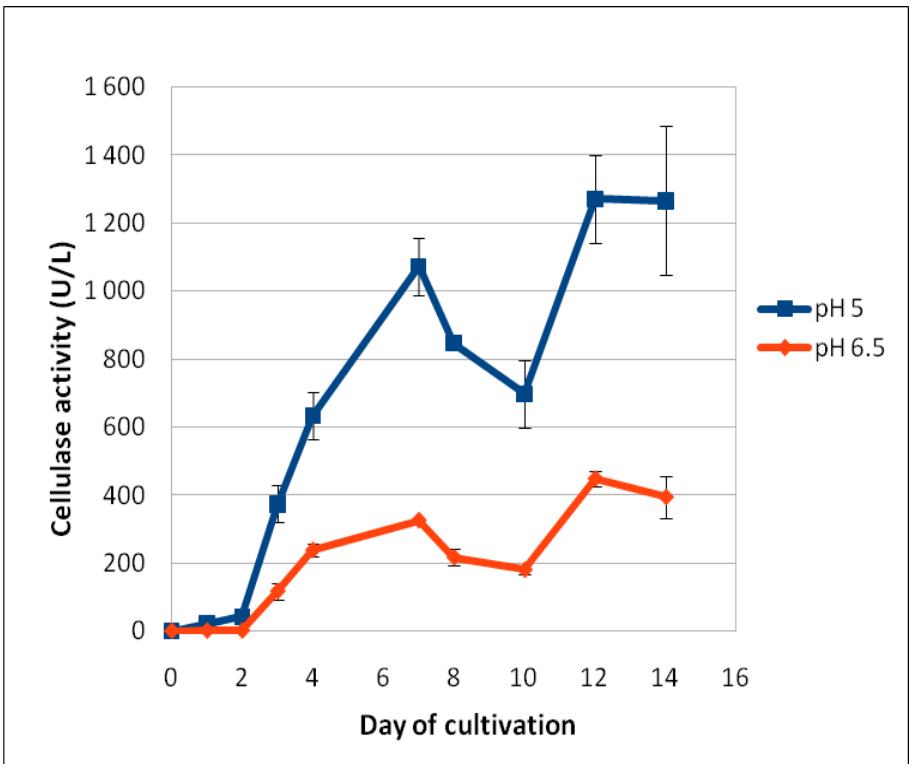

Figure 9: Comparison of the extracellular cellulase activity performed at different pH for *P. pinophilum* grown in birch wood liquid medium with peptone, Unit per litre of growth medium [error bar represent standard deviation of duplicate cultivations]

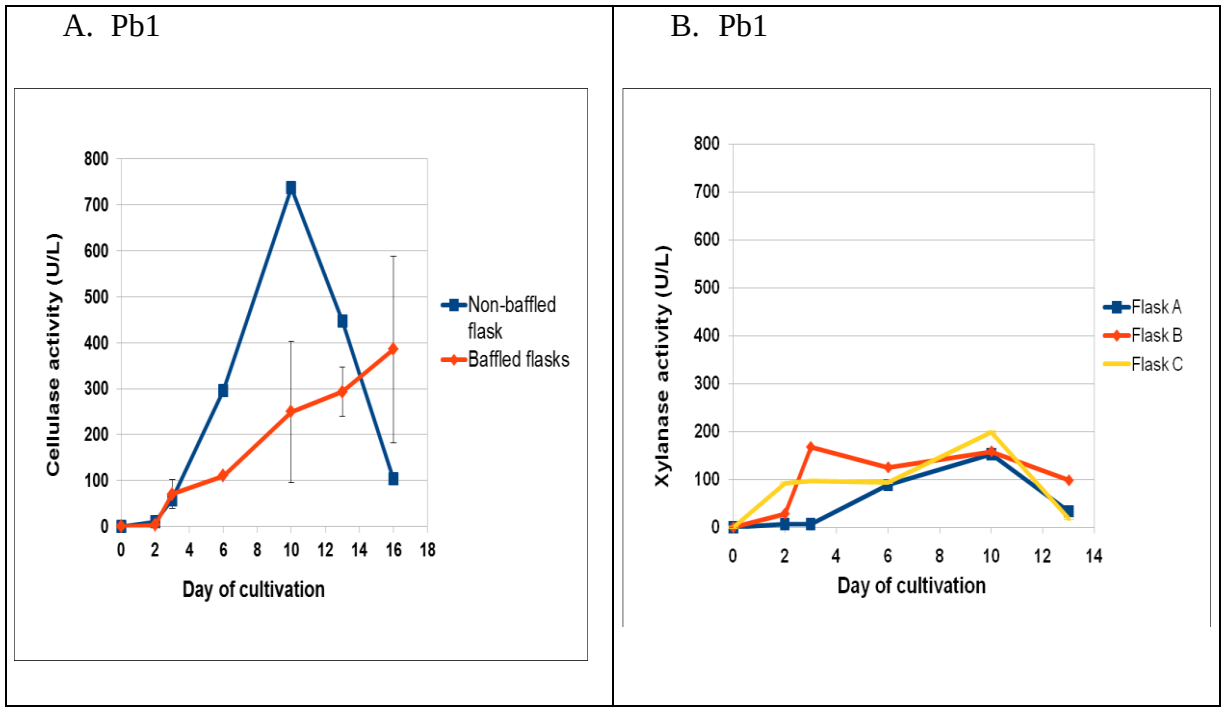

Figure 10: Extracellular cellulase (A) and xylanase (B) activity of *P. pinophilum* grown in birch wood liquid medium, Pb1, Unit per litre of growth medium (Assay performed at pH 6.5) [error bar represent standard deviation of duplicate cultivation]

Enzymes production of Pb1 is shown in Figure 10, and the enzyme activity measurements data and calculations can be found in Appendix IV. The enzyme production was the highest on day 10 of cultivation for both cellulase and xylanase. On the other hand in Figure 11, the specific xylanase activity, which provides the activity of the enzyme per amount g of total protein in the medium, expressed as µmole per miligram protein and minute, or U/mg, is highest on day 2 for flask C and on day 3 for flask B while the specific cellulase activity is highest on day 10 for flask A and C (graph shown in appendix III). Total protein content for all the cultivations can be found in Appendix VI. All three flasks used contained the same composition of growth media where flask A is a 500 ml non-baffled Erlenmeyer flask while flask B and C are baffled flasks. Although the temperature and shaking during the cultivation were the same for all 3 flasks, large differences were still observed in enzyme activities produced. This might be caused by the presence or absence of baffles at the bottom of the flask to increase aeration. Although the shaking speed was the same, the presence of baffles resulted in the transfer of birch wood on the wall surface, which subsequently decreased the wood content in the liquid portion of the medium. Pipetting and absorbance measurement

may also account to the large variation in enzyme activities as it can be seen from large standard deviation in xylanase activity of Flask B on day 3 of cultivation. These are the possible reasons that make the large differences in the result obtained. A larger flask (1 L flask) was used for the cultivation of Pb2 with the decrease in agitation speed to 100 rpm and more precaution in pipetting and measurement were also taken to decrease this kind of variation.

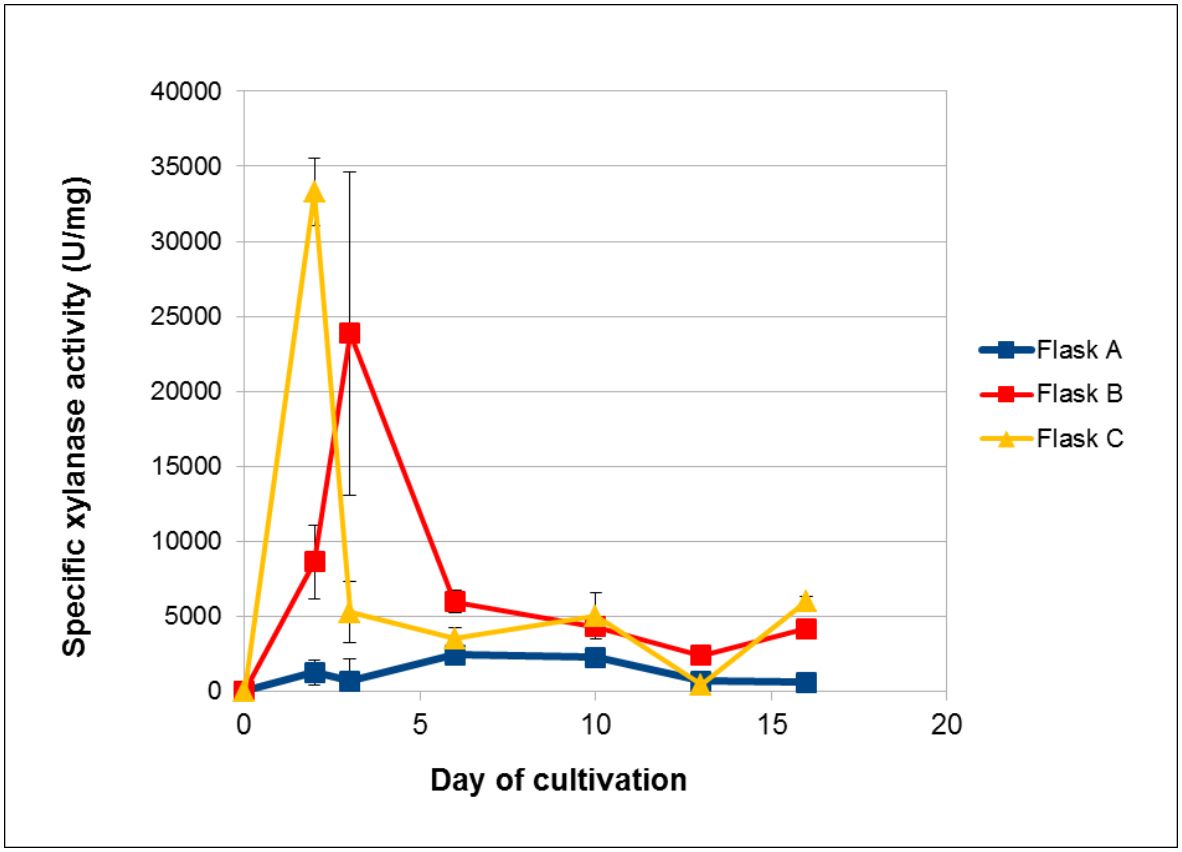

Figure 11: Specific xylanase activity of *P. pinophilum* grown in birch wood liquid medium, Pb1, Unit per mg protein [error bar represents standard deviation of triplicate measurement]

In the previous medium, Pb1, glucose served as the primary energy source while sodium nitrate was the main nitrogen source for the microorganism. In Pb2, in addition to the presence of the aforementioned nitrogen source, peptone was also used, because it has been reported to have been facilitating the cellulolytic enzyme expression (Elisashvili et al. 2008). The cultures were performed in duplicate and the pH of the medium was monitored. The enzyme production in this medium is higher than enzyme produced in birch wood liquid

medium without addition of peptone where the highest cellulase activity was less than 800 U/L.

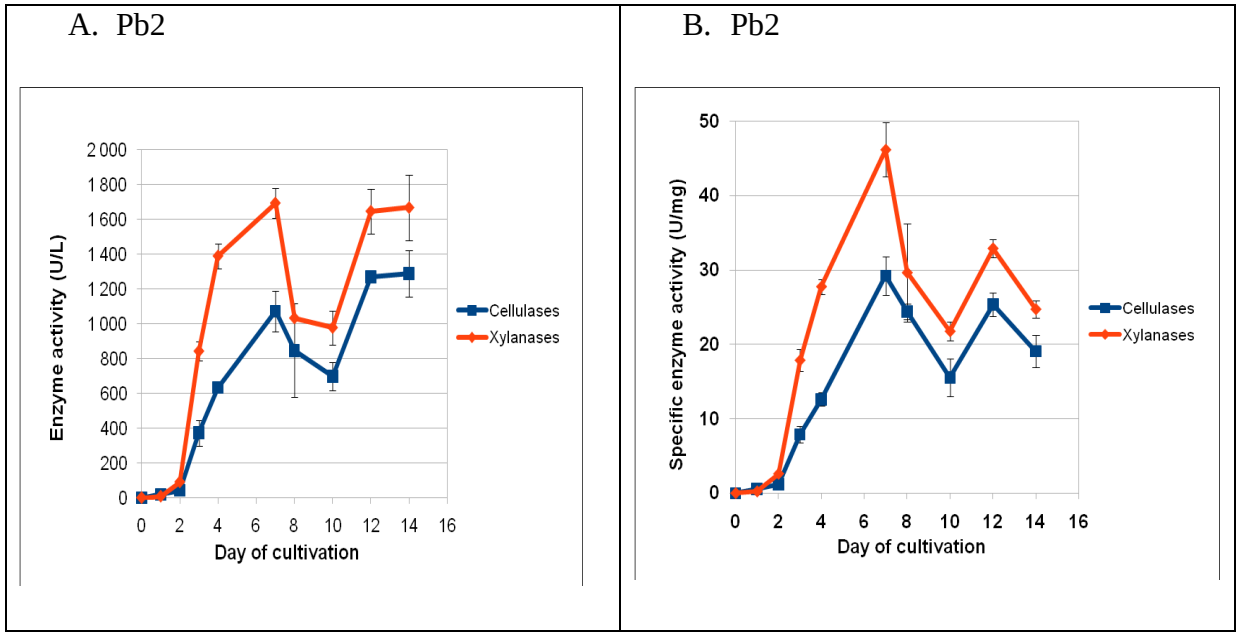

Figure 12: Extracellular cellulase and xylanase activity (A) and specific enzyme activity (B) of *P. pinophilum* grown in birch wood liquid medium with peptone, Pb2 [error bar represent standard deviation of duplicate cultivation]

Cellulase and xylanase activity followed a similar trend, as it is shown in figure 12A. The assays for both enzymes were performed at pH 5; cellulase activity increased to 1072 U/L of medium after 7 days and at the end of cultivation, after 14 days, to 1288 U/L. *P. pinophilum* demonstrated the highest xylanase activity (1693 U/L) at day 7 of cultivation in birch wood liquid medium with peptone, while the highest cellulase activity was exhibited on day 12 and 14 (1271 U/L and 1288 U/L respectively). A drop in enzyme activity was observed on days 8 and 10 for both enzymes. The reason for the sudden decrease in enzyme activity was not clear but it might have been due to the change in the shaking speed or temperature fluctuation during the incubation or sampling error. It is also possible that *P. pinophilum* switch from the production of cellulase and xylanase to other group of enzymes when it detected the change in composition of growth medium. Both the specific activity of xylanase and cellulase is the highest on day 7 (figure 12B). Thus, the addition of peptone in the media increases the enzyme production of *P. pinophilum*.

4.3 Cultivation of Trametes hirsuta on liquid medium

The second microorganism, *T. hirsuta*, displays white colonies when grown on PDA (Figure 13). It grows quickly and the whole agar plate is covered in 4 days at 30^{o}C.

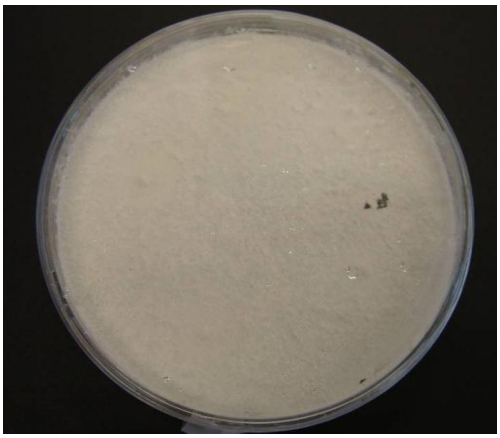

Figure 13: Morphology of *T. hirsuta* on PDA plate

T. hirsuta was cultivated in Tb1 for 2 weeks and extracellular cellulase and xylanase activities were analysed (Figure 14). The highest cellulase activitieswas observed in day 2 of cultivation which confirmed the result from the screening test (section 6.1.1) that *T. hirsuta* is capable of utilizing wood as a carbon source for its growth. As the production of cellulase decreased in day 3, xylanase enzyme production is at its peak. The amount of both the enzymes produced decrease below 100 U/L after 6 day of cultivation. This might due to the probable repression of these enzymes from their products (glucose and xylose) or the microorganisms have stopped growing. The large differences in the amount of enzymes produced in different flasks might have been caused by the difference in flasks (baffled and non-baffled) and the transfer of wood substrate onto the flasks wall due to fast shaking.

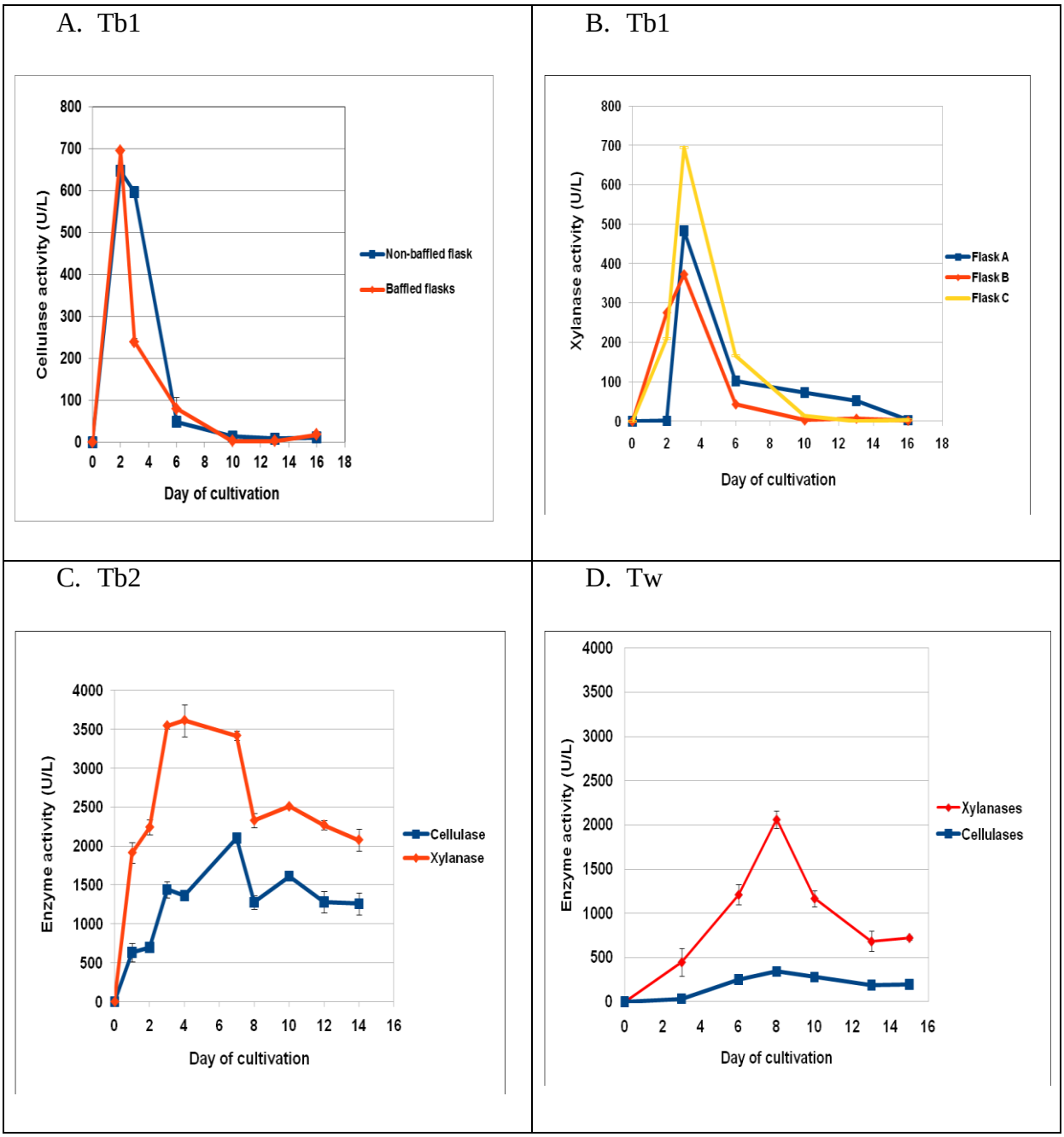

Figure 14: Enzyme activity of *T. hirsuta* in liquid cultivation, Unit per litre of growth medium. (A) Cellulase activity of Tb1. (B) Xylanase activity of Tb1. (C) Cellulase and xylanase activity of Tb2. (D) Enzyme activity of Tw [error bar represents standard deviation of duplicate cultivation in (C) and triplicate cultivation in (D)]

Cultures were performed in duplicate in medium Tb2 and the flasks were being checked regularly to make sure that the medium was homogenous. Also, the pH of the medium was monitored. With the starting medium pH of 5.7, *T. hirsuta* changed the pH towards more

neutral values (around pH 6.5-7) unlike *P. pinophilum*, which changed the pH to more acidic. The production of extracellular cellulase and xylanase were analysed at pH 6.5 and the results were shown in Figure 14.

The cellulase and xylanase activity of *T. hirsuta* was higher with the addition of peptone in the liquid medium; more than 2000 U/L and 3500 U/L respectively (Figure 14C) as peptone served as a protein and amino acid source that induces the microbial growth. The enzyme production was high in the first 7 days of cultivation, and decreased afterwards. The cellulases and xylanases activities of *T. hirsuta* in wheat bran liquid cultivation (Tw) was also higher than Tb1 and the highest activity was observed on day 8 of cultivation (Figure 14D).

4.4 Cultivation on solid state culture

T. hirsuta was cultivated in SSC, using wheat bran at 90% relative humidity as nutrient source for a total of 22 days (Figure 15)

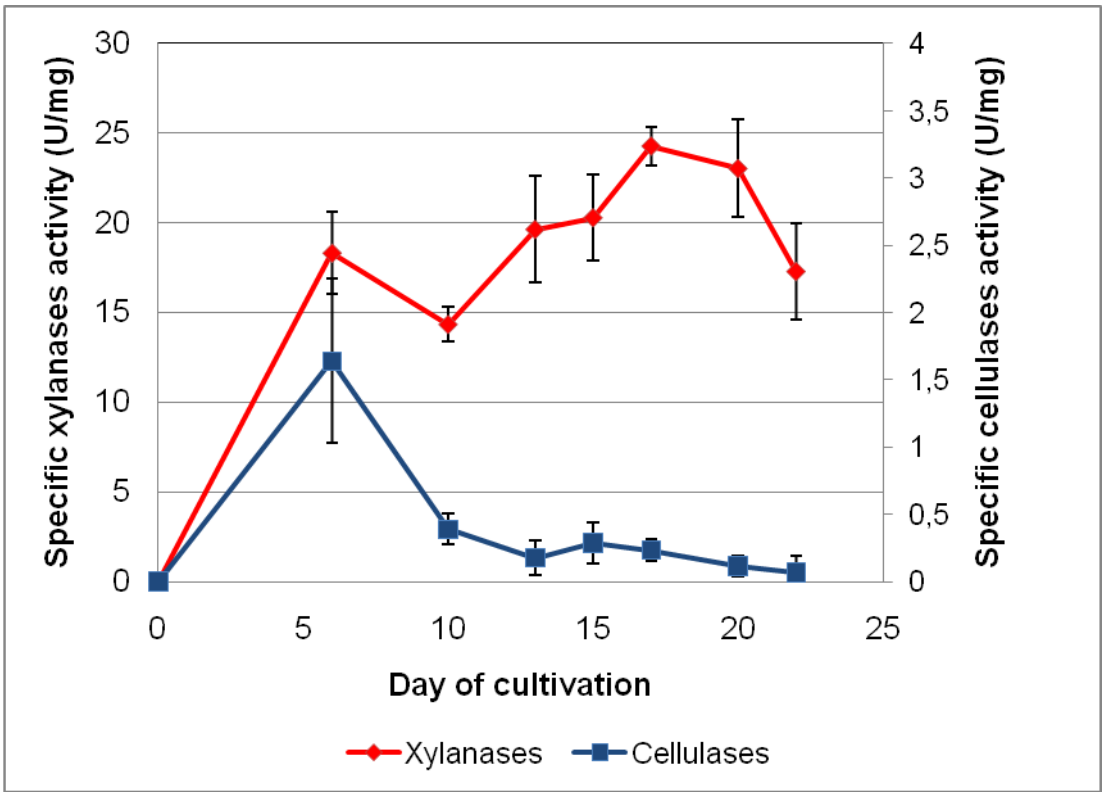

Figure 15: Specific cellulase and xylanase activity of of *T. hirsuta* in 90% relative humidity wheat bran SSC (SSCTw1) in Units per mg of total protein [error bar represents standard deviation of triplicate cultivation]

The production of cellulase was the highest on day 6, followed by a large decrease afterwards. In the contrary, the xylanase activity was continuously increasing until day 17. Moreover, laccase and manganese peroxidase presence was also monitored, but no activity was detected. Thus, sample of day 17, with the highest xylanase activity, was concentrated 10 times and analyzed but there was no detectable activity for laccase. Considering that laccase activity was expected from *T. hirsuta* (Couto et al. 2006 and Böhmer et al. 2011), its absence was attributed to the contamination of the cultures – an explanation enhanced by the green mycelia present on the medium (instead of the usual white colonies of *T. hirsuta*). Thus, the cultivation of *T. hirsuta* in SSC wheat bran was repeated (SSCTw2 – Figure 16). The specific

activity of cellulase was quite constant for the whole period of cultivation at 3 U/mg $_{protein}$ while the specific activity of xylanase was higher (5.93 U/mg protein) on day 7.

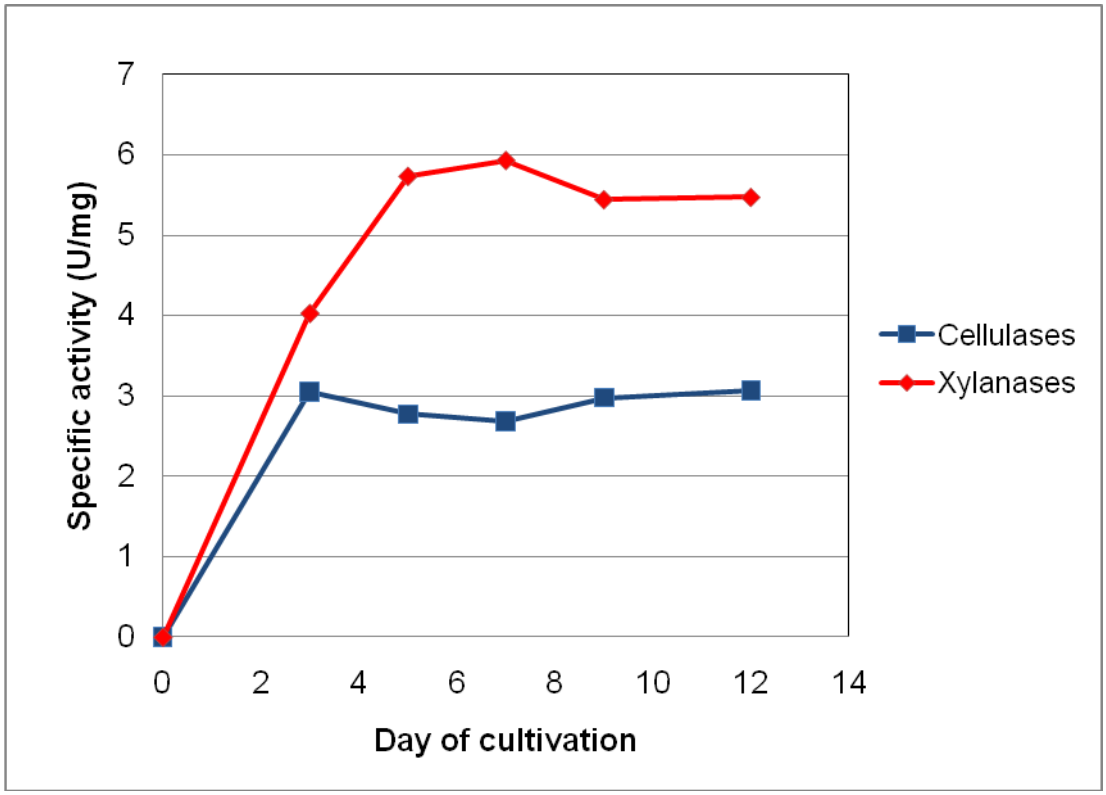

Figure 16: Specific cellulase and xylanase activity of *T. hirsuta* in 90% relative humidity wheat bran SSC (SSCTw2), Unit per mg of total protein

SSCTw2 cultivation also induced the production of laccase (Figure 17). The highest production was on day 5 of cultivation (1081 U/L) and the amount of laccase produced decreased until it was not detectable on day 12. The measurement data and calculation of laccase activity is given in Appendix V. The expression of another lignin-degrading enzyme, manganese peroxidase, was also examined but no activity was detected.

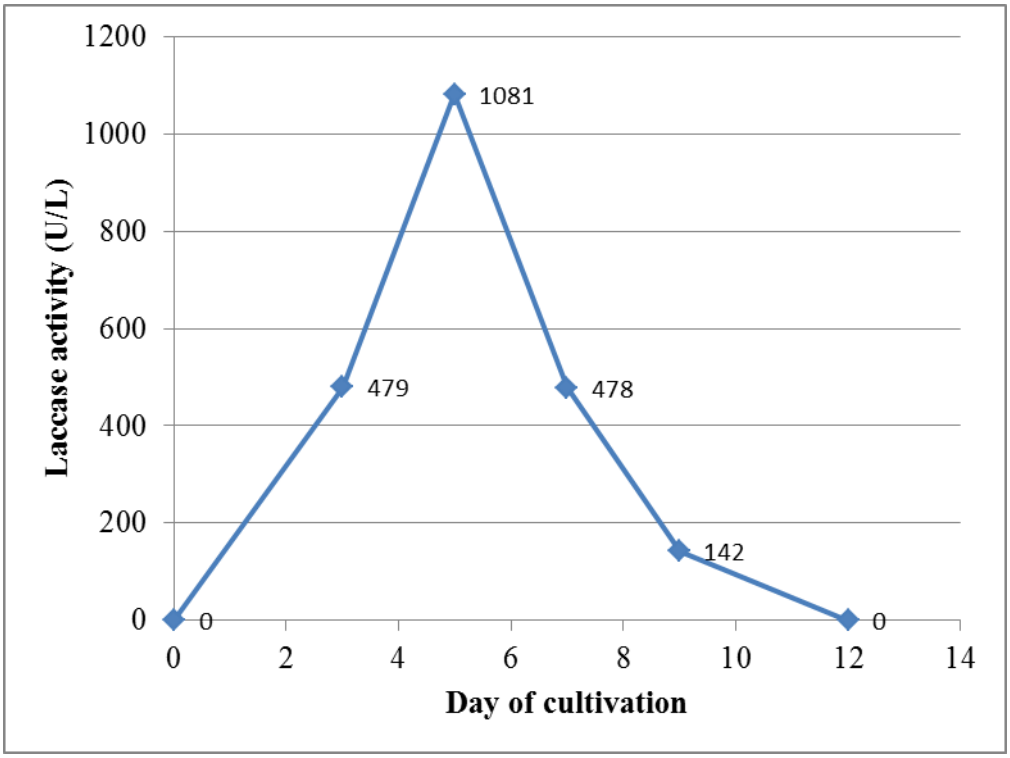

Figure 17: Laccase activity of SSCTw2 in unit per liter

T. hirsuta was also inoculated on birch wood in solid state cultures, however, no visible growth was observed and no cellulose or xylanase activity was detected. Birch wood might be less accessible for the organisms to start growing compared to wheat bran which is rich in nutrients, the existence of loose texture in moist conditions and a large surface area, or maybe the conditions were not optimal [moisture, temperature](Chandra et al. 2007). Thus, wheat bran is more suitable for SSC and for the production of lignocellulose degrading enzymes.

The highest activities of the enzymes monitored are shown in Table 6, expressed in units per mg of total protein (U/mg) during cultivation of each microorganism in different substrates. The cellulase and xylanse produced were higher for both microorganisms in birch2 culture, in the presence of peptone in the culture medium. Wheat bran liquid cultivation induced higher xylanase production in *T. hirsuta* from 26 $U/g_{substrate}$ in birch wood liquid medium without peptone to 103 $U/g_{substrate}$. SSC birch was unable to induce neither production nor growth of the microorganisms while SSC wheat induced the enzyme production of 18 $U/g_{substrate}$ of cellulase and 36 $U/g_{substrate}$ of xylanase.

Table 5 presents the comparison of cellulase and xylanse produced per gram of substrate in different cultures. The results suggest the increase in enzyme production where there was an

addition of peptone to the birch wood liquid medium. The cellulase and xylanase activity of *T. hirsuta* in SSCw2 (3595 U/L and 36 U/L respectively) is higher than in b2 cultivation (1694 U/L and 85 U/L respectively) but the enzyme activities per gram substrate were lower.

Table 5: Cellulase and xylanase activities expressed in units per gram of substrates

Strain	Culture	Cellulase (U/g substrate)	Xylanase (U/g substrate)
P. pinophilum	Pb1	21	8
	Pb2	63	85
T. hirsuta	Tb1	34	26
	Tb2	105	180
	Tw	17	103
	SSCTb	0	0
	SSCTw2	18	36

5. Discussion

This project aimed at identifying strains of filamentous fungi with high potential in the degradation of lignocellulosic substrate. Seven different fungal strains were screened on PDA media containing colored indicator compounds that enable the visual detection of ligninolytic enzymes production. The use of colored indicators is generally simpler because there is no requirement for sample handling and measurement (Kalmiş et al. 2008). Several different compounds have been used as indicators for ligninolytic enzymes production. RBBR and guaiacol are used frequently and their results correlate well to each other (Kiiskinen et al. 2004 and Ang et al. 2011). Some fungal strains tested in the present study gave positive test results in RBBR containing medium but failed to decolorize guaiacol might be due to several reasons. One of them could be because the fungi require a longer adaptation time to produce ligninolytic enzymes for the decolorization of guaiacol dye, as it has been reported for other colour indicators, such as RBBR (Ang et al. 2011). Methyl green was also used in this project for ligninolytic enzymes screening. The lack of detection of growth for *T. hirsuta* and *P. pinophilum* on methyl green containing PDA media could be attributed to the high concentration of the indicator, which might have an inhibitory effect (Eichlerová et al. 2006).

Two fungal strains showing positive reactions in the screening in birch wood solid medium and dye decolorization assay were grown in different lignocellulosic materials, namely birch wood and wheat bran, to induce the production of lignocelluloses-degrading enzymes by *P. pinophilum* and *T. hirsuta*. Wheat bran has been used as substrate in most of the studies for the production of different enzymes such as cellulase, xylanase, ligninolytic enzymes, etc (Chandra et al. 2007 and Souza et al. 2006). Cellulase and xylanase activities were detected in all the samples except in SSC birch and they were used to follow the growth of the organisms during the cultivation. The increase in the enzyme content in the media is an indication of the fungal growth, while, the decrease might indicate that the organisms had stop growing or that the expression of this specific enzyme has been repressed by its respective enzymatic reaction products.

Among the lignocellulose degrading enzyme, *P. pinophilum* IBT10872 produced cellulase and xylanase although no lignin-degrading enzymes were detected in birch wood samples. The cellulases activity was 0.4 U/ml which is comparable with the results from Krogh

(2004), who detected 0.32 U/ml of FPA of the same strain growth on cellulose. *P. pinophilum* TERIDB1 was shown to be able to produce three lignin-degrading enzymes namely laccase, MnP and LiP on corn cobs molasses and wheat straw (Pant and Adholeya 2007). These lignin-degrading enzymes were not detected in the samples of the present study, possibly because it was a different strain than the one reported and the substrate used were different *T. hirsuta* NCIM1201 produced higher amount of enzymes compared to *P. pinophilum* while both microorganisms produced higher amount of cellulase and xylanase in birch2 cultivation when peptone was added as additional nitrogen source than birch1 cultivation without peptone. The supplementation of peptone to control medium has also been shown to give twofold increase in CMCase and xylanase activity by *Coriolopsis polyzona*. The positive effect of additional nitrogen on enzyme activity has been attributed to the higher biomass yield (Elisashvili et.al. 2008).

SSC has been found to induce the production of ligninolytic enzyme by *Pleurotus pulmonarius*, as it mimics the natural habitat of the fungus and holds great potential for the production of enzymes including laccase and MnP. Wheat bran contains several phenolic compounds which are efficient laccase inducers and the use of wheat bran in SSC of *P. pulmonarius* appeared to be an excellent growth and laccase inducer (Souza et.al. 2005). As it has been reported in the literature that *T. hirsuta* produces laccase, SSC cultures in wheat bran and birch wood were performed to examine these findings (Couto et al. 2006 and Böhmer et al. 2011). Cellulase and xylanase produced were higher in SSC than in liquid cultivation. The enzyme activity, however, per gram of substrate is almost 3 times higher in liquid cultivation with peptone than SSC taking into account that the amount of substrate is five times higher in SSC as compared to liquid medium. This is due to the high moisture content in SSC that reduces the porosity of the substrate limiting the oxygen transfer, and thus resulting in only surface growth and limiting the growth within the whole surface (Souza et al. 2006). Apart from the two hydrolytic enzymes, an oxidative enzyme, laccase, was also produced in SSCTw2. The amount of laccase produced was high (1081 U/L) compared to amount produced by *T. hirsuta* (BT2566) in solid state fermentation of orange peel (207 U/L) while laccase production has been shown to be 2000 U/L in expanded bed reactor (Böhmer et al. 2011 and Couto et al. 2006). The enzyme activity is species dependent and the type of substrate used and cultivation method also affect the production (Elisashvili et al. 2008). No growth or enzyme production was detected in SSCb due to availability and low accessibility of nutrient for fungal growth.

6. Conclusion

T. hirsuta and *P. pinophilum* were chosen for studies from seven fungal strains that were screened for their grown ability on birch solid medium and synthetic dye decolorization. The cellulases and xylanases activities of both fungi in different culture conditions were monitored and followed as a growth indicator. This study shows that the use of different carbon sources result in differences in the amount of certain enzymes produced. The addition of peptone in the medium clearly increases the cellulases and xylanases produced. Growth in solid state cultures on wheat bran not only induced the production of higher amount of these enzymes but also induces the production of the lignin-degrading enzyme, laccase, while birch wood SSC wasn't a suitable growth medium for *T. hirsuta*. In conclusion, wheat bran seems more suitable than birch wood as enzyme inducer. However, growth conditions should be optimized in order to achieve a larger variety and detectable activities of the biomass degrading enzymes of interest.

7. References

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8. Appendix

Appendix I: Morphology of filamentous fungi on PDA

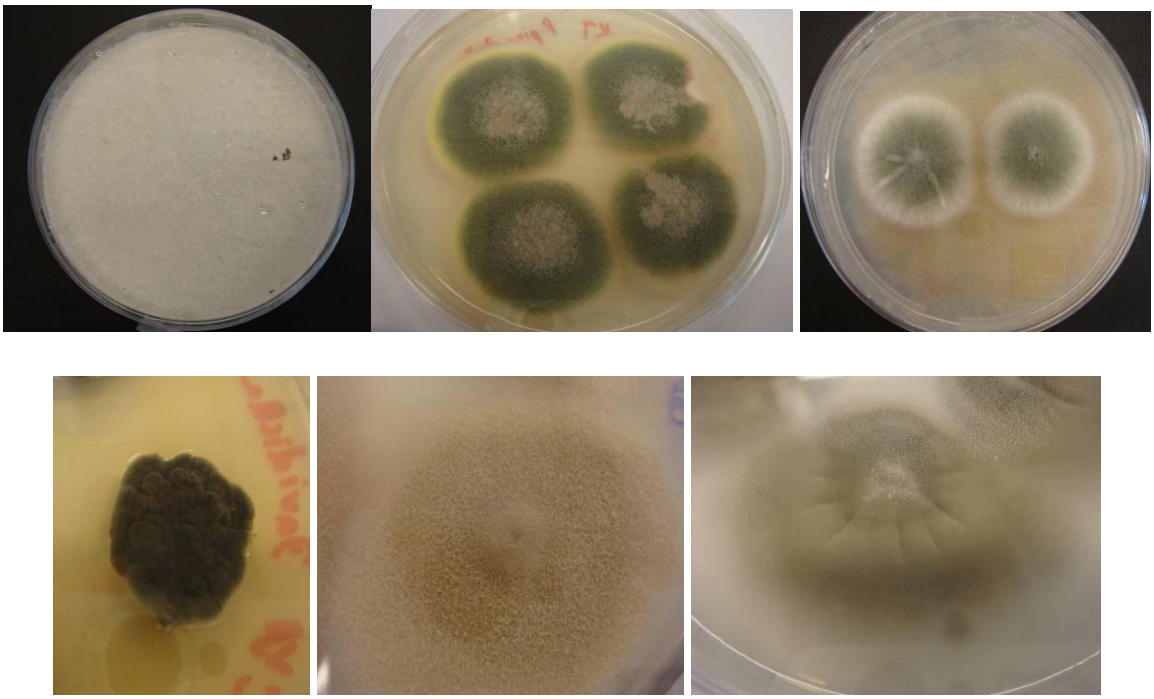

Figure 18: Morphology of fungi on PDA medium (From left to right – *T. hirsuta, P. pinophilum, S. polyspora, D. tassiana, U. maydis* and *P. Thomii)*

Appendix II: Medium pH of fungi growth in birch wood liquid medium with peptone

Table 6: Medium pH of *T. hirsuta* and *P. pinophilum* in birch wood liquid medium with peptone

Day of cultivation	*Trametes hirsuta*		*Penicillium pinophilum*	
	Flask A	Flask B	Flask A	Flask B
0	5.7	5.7	5.7	5.7
2	6.59	6.4	4.72	4.7
3	6.02	6.03	4.81	4.97
4	6.66	6.8	5.09	5.05
7	7.06	7.13	5.03	5.06
8	7.01	7.14	4.93	5.01
10	6.95	7.08	4.77	4.91
12	6.91	7.03	4.8	4.9
14	6.85	6.98	4.9	4.95
16	6.74	6.92	4.92	5

Appendix III: Specific cellulase activity of P. pinophilum in birch wood liquid medium without peptone

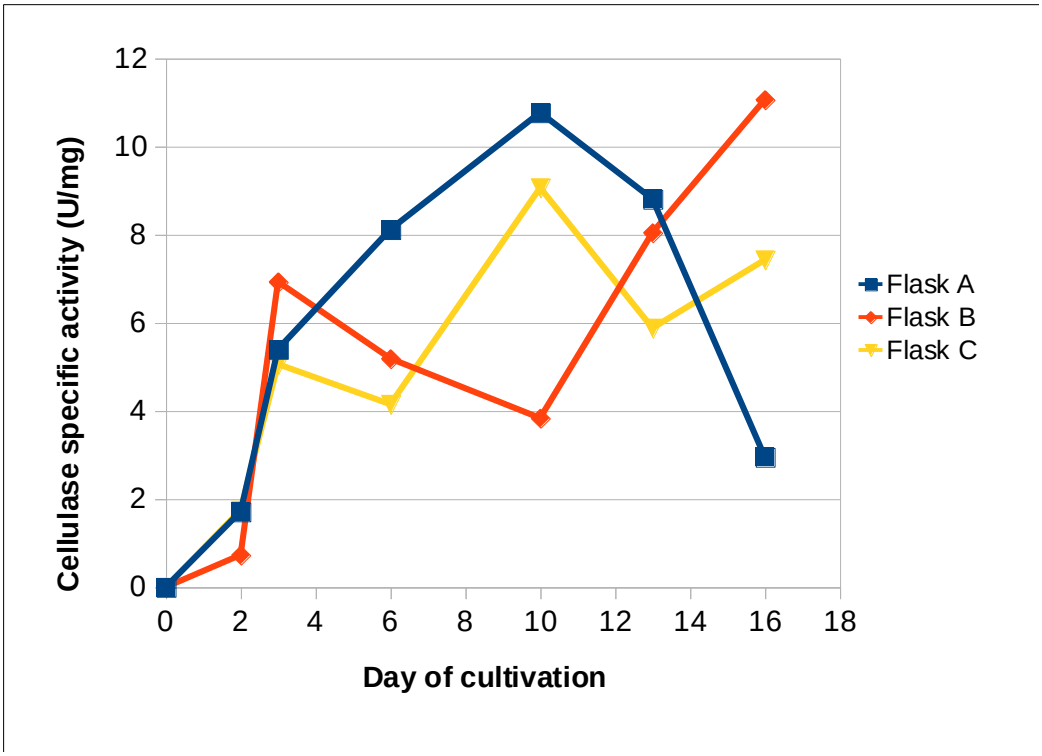

Figure 19: Specific cellulase activity of *P. pinophilum* in birch wood liquid medium without peptone, unit for mg of total protein

Appendix IV: Calculations for cellulases and xylanases activity

Standard curve for DNS reagent

Table 7: Absorbance at 540 nm of glucose and xylose solution at different concentrations

	g/L	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Concentration	µmole/L	252.3	504.6	1009.2	1513.9	2018.5	2523.1	3027.7	3532.3	4036.9	4541.6	5046.2
Glucose	OD 540 (DNS 1)	-	0.039	0.105	0.182	0.258	0.338	0.413	0.488	0.548	0.638	0.704
	OD 540 (DNS 2)	0.009	0.036	0.104	0.180	0.257	0.328	0.401	0.471	0.547	0.622	0.691
Xylose	OD 540 (DNS 1)	-	0.047	0.131	0.223	0.327	0.407	0.493	0.563	0.672	0.748	0.829
	OD 540 (DNS 2)	0.013	0.050	0.136	0.235	0.323	0.422	0.525	0.588	0.682	0.770	0.845

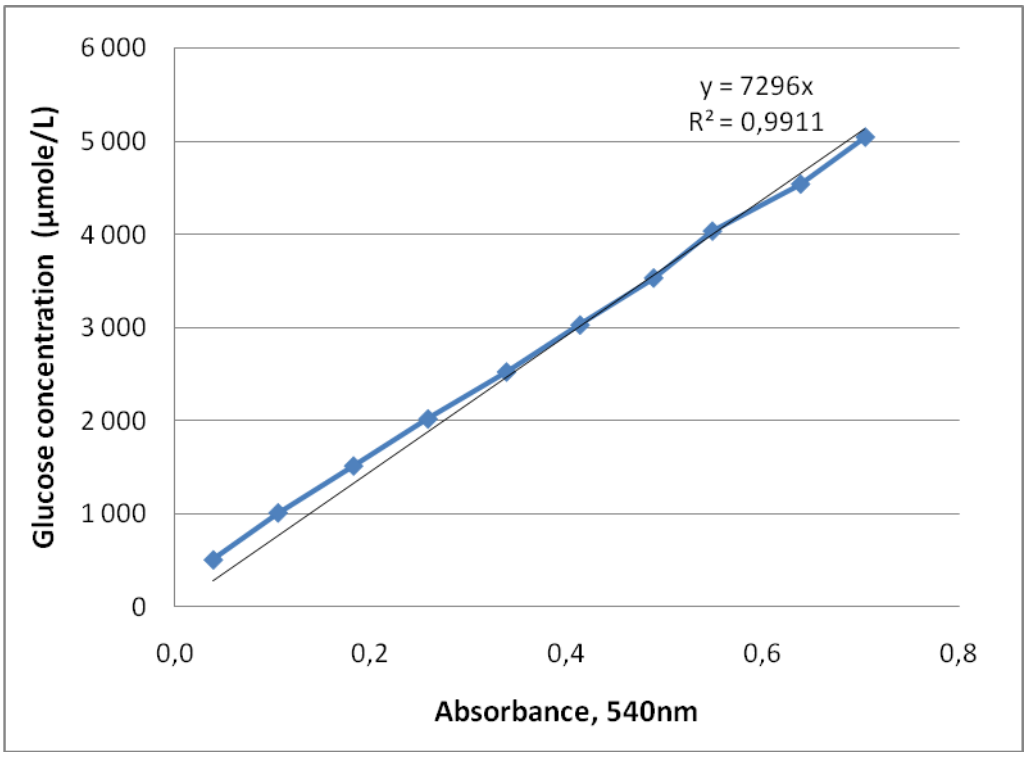

Figure 20: Glucose standard curve for DNS reagent 1

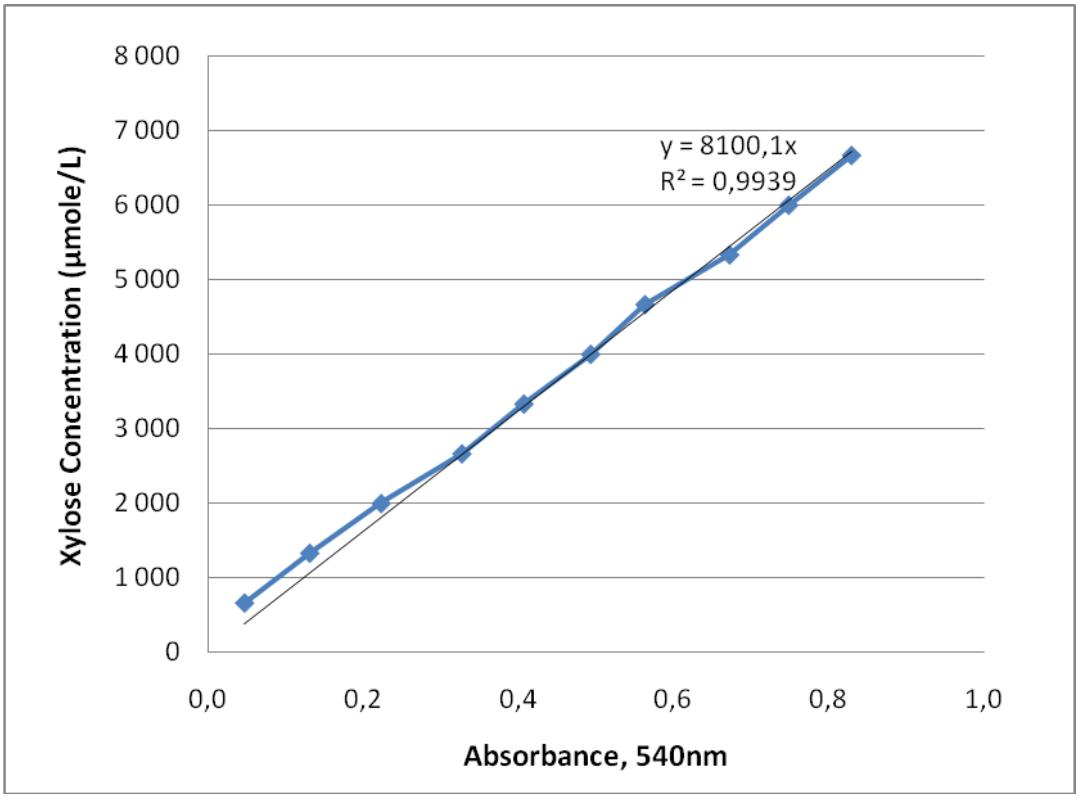

Figure 21: Xylose standard curve for DNS reagent 1

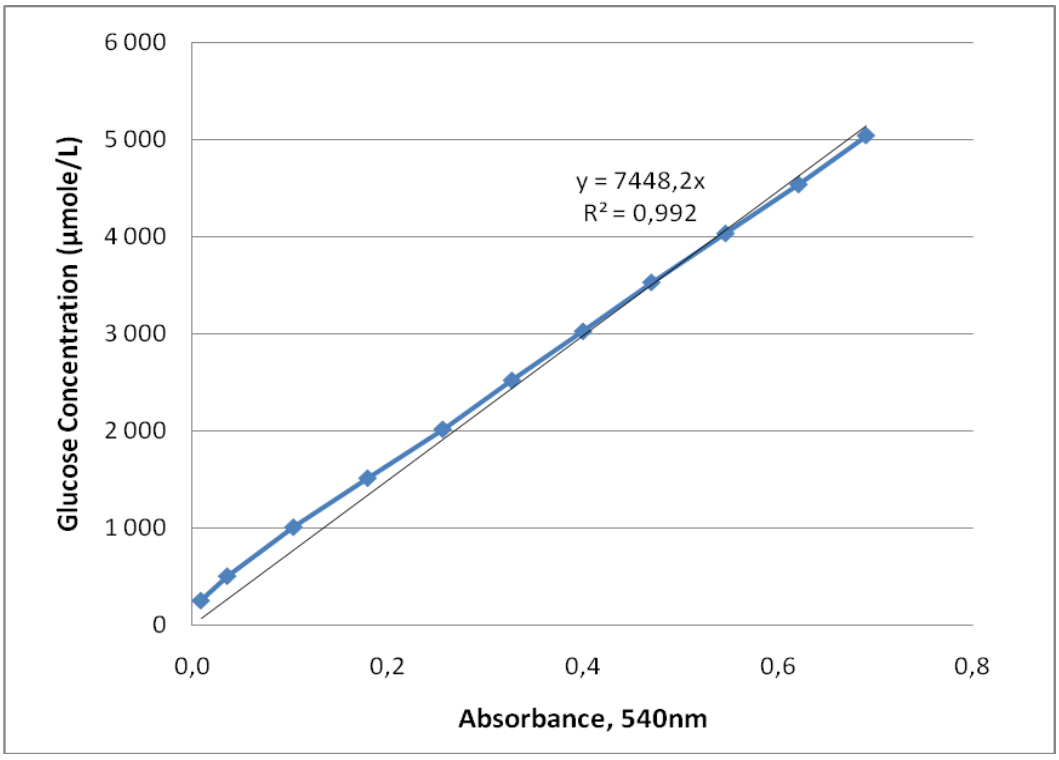

Figure 22: Glucose standard curve for DNS reagent 2

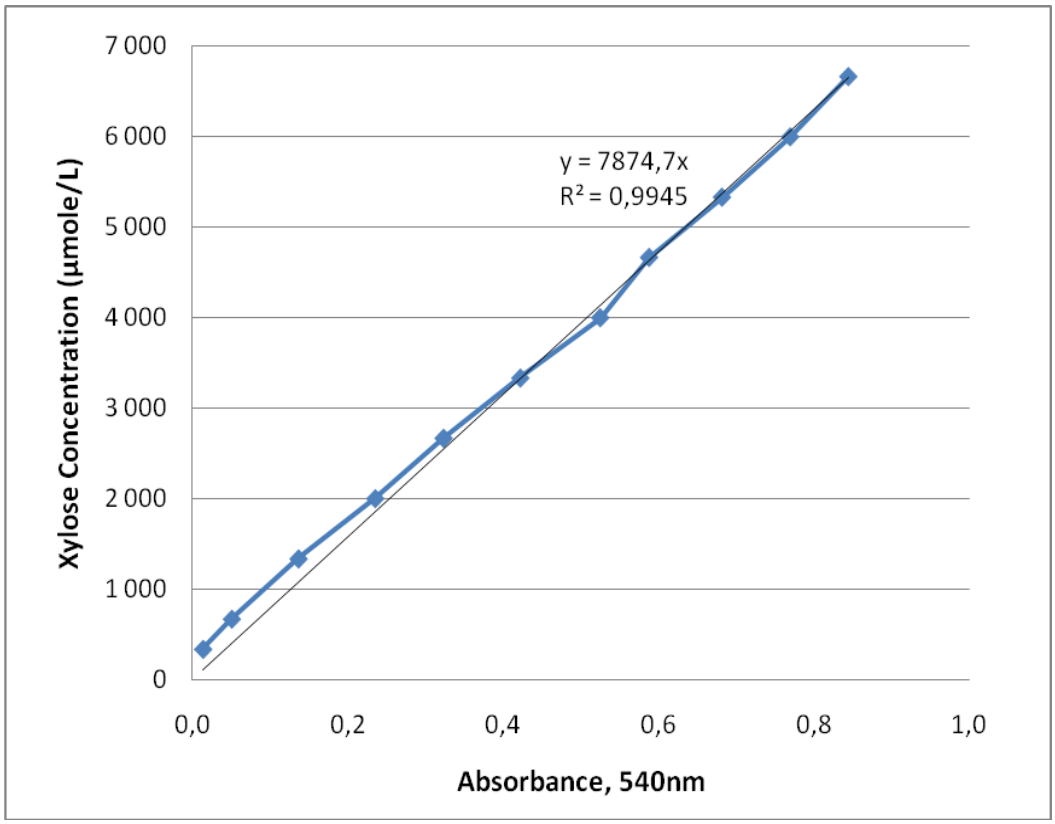

Figure 23: Xylose standard curve for DNS reagent 2

Calculation for cellulases and xylanases activity

Cellulases activity of day 6 flask A of *P. pinophilum* growth in birch liquid medium

Average control OD = 0.003

Average sample (enzyme supernatant) OD = 0.064

Corrected OD = Average sample OD – average control OD

= 0.064 – 0.003

= 0.061

Enzyme activity (Glucose equivalent content)

µmole/L of reaction mixture= corrected OD x slope of standard curve

= 0.061 x 7296

= 445.06 µmole/L of reaction mixture

µmole/L culture filtrate = 445.06 x $\frac{\text{volume of reaction mixture}}{\text{volume of sample}}$

= 445.06 x (200/20)

= 4450.6 µmole/L culture filtrate

Sample volume were 20 ul for all samples except if specifically stated (eg. xylanase in SSC wheat)

Enzyme activity (Unit/L) = 4450.6 / incubation time (reaction time)

= 4450.6/15

= 296.70 µmole/L.min (U/L)

Appendix IV: Calculations for cellulases and xylanases activity

Table 8: Absorbance at 540 nm for cellulase activity of Pb1

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
2	A	10	0.002	0.002	0.001	0.002	0.002	0.003	0.004	0.003	0.001
		20	0.001	0.002	0.002	0.002	0.003	0.003	0.002	0.003	0.001
	B	10	0.039	0.036	0.037	0.037	0.038	0.038	0.037	0.038	0.000
		20	0.037	0.036	0.038	0.037	0.036	0.036	0.035	0.036	-0.001
	C	10	0.072	0.063	0.070	0.068	0.070	0.068	0.069	0.069	0.001
		20	0.070	0.065	0.070	0.068	0.070	0.072	0.069	0.070	0.002
3	A	10	0.001	0.002	0.001	0.001	0.009	0.009	0.010	0.009	0.008
		20	0.005	0.005	0.004	0.005	0.029	0.030	0.032	0.030	0.026
	B	10	0.001	0.002	0.001	0.001	0.007	0.008	0.009	0.008	0.007
		20	0.005	0.006	0.005	0.005	0.026	0.030	0.027	0.028	0.022
	C	10	0.001	0.001	0.002	0.001	0.012	0.015	0.015	0.014	0.013
		20	0.004	0.004	0.004	0.004	0.037	0.040	0.040	0.039	0.035
6	A	15	0.003	0.004	0.003	0.003	0.064	0.060	0.069	0.064	0.061
		30	0.016	0.016	0.017	0.016	0.138	0.134	0.136	0.136	0.120
	B	15	0.003	0.002	0.003	0.003	0.024	0.026	0.025	0.025	0.022
		30	0.008	0.005	0.006	0.006	0.051	0.057	0.051	0.053	0.047
	C	10	0.002	0.003	0.002	0.002	0.017	0.018	0.018	0.018	0.015
		20	0.003	0.004	0.003	0.003	0.035	0.037	0.035	0.036	0.032
10	A	10	0.003	0.004	0.003	0.003	0.100	0.106	0.107	0.104	0.101
		20	0.008	0.008	0.007	0.008	0.193	0.190	0.194	0.192	0.185
	B	10	0.003	0.003	0.003	0.003	0.023	0.022	0.022	0.022	0.019
		20	0.004	0.003	0.005	0.004	0.051	0.047	0.050	0.049	0.045
	C	10	0.003	0.004	0.004	0.004	0.052	0.053	0.053	0.053	0.049
		20	0.004	0.004	0.004	0.004	0.100	0.105	0.104	0.103	0.099
13	A	10	0.003	0.005	0.005	0.004	0.067	0.065	0.065	0.066	0.061
		20	0.006	0.007	0.005	0.006	0.125	0.131	0.129	0.128	0.122
	B	10	0.001	0.002	0.001	0.001	0.048	0.047	0.045	0.047	0.045
		20	0.002	0.001	0.002	0.002	0.092	0.087	0.090	0.090	0.088
	C	10	0.001	0.003	0.004	0.003	0.027	0.032	0.028	0.029	0.026
		20	0.004	0.004	0.005	0.004	0.072	0.074	0.077	0.074	0.070
16*	A	10	0.003	0.003	0.004	0.003	0.016	0.017	0.019	0.017	0.014
		20	0.004	0.004	0.004	0.004	0.034	0.035	0.036	0.035	0.031
	B	10	0.006	0.005	0.005	0.005	0.073	0.075	0.081	0.076	0.071
		20	0.011	0.010	0.009	0.010	0.151	0.153	0.160	0.155	0.145
	C	10	0.009	0.010	0.010	0.010	0.037	0.032	0.033	0.034	0.024
		20	0.008	0.011	0.010	0.010	0.073	0.074	0.077	0.075	0.065

Table 9: Calculation for cellulase activity of Pb1

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
2	A	10	0.001	9.73	97.28	9.73
		20	0.001	7.30	72.96	3.65
	B	10	0.000	2.43	24.32	2.43
		20	-0.001	-9.73	-97.28	-4.86
	C	10	0.001	4.86	48.64	4.86
		20	0.002	14.59	145.92	7.30
3	A	10	0.008	58.37	583.68	58.37
		20	0.026	187.26	1872.64	93.63
	B	10	0.007	48.64	486.40	48.64
		20	0.022	162.94	1629.44	81.47
	C	10	0.013	92.42	924.16	92.42
		20	0.035	255.36	2553.60	127.68
6	A	15	0.061	445.06	4450.56	296.70
		30	0.120	873.09	8730.88	291.03
	B	15	0.022	162.94	1629.44	108.63
		30	0.047	340.48	3404.80	113.49
	C	10	0.015	111.87	1118.72	111.87
		20	0.032	235.90	2359.04	117.95
10	A	10	0.101	736.90	7368.96	736.90
		20	0.185	1347.33	13473.28	673.66
	B	10	0.019	141.06	1410.56	141.06
		20	0.045	330.75	3307.52	165.38
	C	10	0.049	357.50	3575.04	357.50
		20	0.099	722.30	7223.04	361.15
13	A	10	0.061	447.49	4474.88	447.49
		20	0.122	892.54	8925.44	446.27
	B	10	0.045	330.75	3307.52	330.75
		20	0.088	642.05	6420.48	321.02
	C	10	0.026	192.13	1921.28	192.13
		20	0.070	510.72	5107.20	255.36
16*	A	10	0.014	104.27	1042.75	104.27
		20	0.031	230.89	2308.94	115.45
	B	10	0.071	528.82	5288.22	528.82
		20	0.145	1077.51	10775.06	538.75
	C	10	0.024	181.24	1812.40	181.24
		20	0.065	484.13	4841.33	242.07

Note: slope value = 7296 except for * = 7448.2

Appendix IV: Calculations for cellulases and xylanases activity

Table 10: Absorbance at 540 nm for xylanase activity of Pb1

Day	Sample	Incubation time (min)	Control			Average	Enzyme supernatant			Average	Corrected OD
			1	2	3		1	2	3		
2	A	30	0.010	0.012	0.009	0.010	0.012	0.015	0.012	0.013	0.003
	B	30	0.059	0.059	0.056	0.058	0.068	0.072	0.066	0.069	0.011
	C	30	0.097	0.090	0.092	0.093	0.126	0.130	0.126	0.127	0.034
3	A	30	0.012	0.008	0.023	0.014	0.023	0.011	0.017	0.017	0.003
	B	30	0.005	0.006	0.009	0.007	0.096	0.070	0.040	0.069	0.062
	C	30	0.020	0.019	0.015	0.018	0.043	0.049	0.069	0.054	0.036
6	A	30	0.005	0.001	0.001	0.002	0.038	0.037	0.030	0.035	0.033
	B	30	0.008	0.009	0.011	0.009	0.050	0.062	0.055	0.056	0.046
	C	30	0.003	0.000	0.001	0.001	0.042	0.028	0.039	0.036	0.035
10	A	30	0.006	0.010	0.021	0.012	0.064	0.077	0.066	0.069	0.057
	B	30	0.005	0.012	0.008	0.008	0.063	0.066	0.072	0.067	0.059
	C	30	0.006	0.006	0.009	0.007	0.090	0.055	0.096	0.080	0.073
13	A	30	0.000	0.000	0.000	0.000	0.007	0.012	0.018	0.012	0.012
	B	30	0.006	0.005	0.008	0.006	0.048	0.032	0.048	0.043	0.036
	C	30	0.008	0.005	0.008	0.007	0.015	0.017	0.010	0.014	0.007
16*	A	30	0.009	0.010	0.007	0.009	0.015	0.016	0.018	0.016	0.008
	B	30	0.010	0.011	0.010	0.010	0.085	0.085	0.083	0.084	0.074
	C	30	0.010	0.015	0.012	0.012	0.065	0.070	0.065	0.067	0.054

Table 11: Calculation for Xylanase activity of Pb1

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
2	A	30	0.003	21.60	216.00	7.20
	B	30	0.011	86.40	864.01	28.80
	C	30	0.034	278.10	2781.03	92.70
3	A	30	0.003	21.60	216.00	7.20
	B	30	0.062	502.21	5022.06	167.40
	C	30	0.036	288.90	2889.04	96.30
6	A	30	0.033	264.60	2646.03	88.20
	B	30	0.046	375.30	3753.05	125.10
	C	30	0.035	283.50	2835.04	94.50
10	A	30	0.057	459.01	4590.06	153.00
	B	30	0.059	475.21	4752.06	158.40
	C	30	0.073	594.01	5940.07	198.00
13	A	30	0.012	99.90	999.01	33.30
	B	30	0.036	294.30	2943.04	98.10
	C	30	0.007	56.70	567.01	18.90
16*	A	30	0.008	60.36	603.57	20.12
	B	30	0.074	582.58	5825.80	194.19
	C	30	0.054	427.75	4277.50	142.58

Note: slope value = 8100.1 except for * = 7872.7

Table 12: Absorbance at 540 nm for Cellulase activity of Pb2 (essay in pH 6.5)

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
1	A	15	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000
		30	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	15	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	-0.000
		30	0.001	0.001	0.002	0.001	0.001	0.000	0.001	0.001	-0.001
2	A	15	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.000
		30	0.000	0.002	0.002	0.001	0.003	0.002	0.002	0.002	0.001
	B	15	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.000
		30	0.001	0.001	0.001	0.001	0.004	0.004	0.004	0.004	0.003
3	A	15	0.002	0.001	0.002	0.002	0.021	0.023	0.023	0.022	0.021
		30	0.002	0.002	0.002	0.002	0.050	0.049	0.048	0.049	0.047
	B	15	0.001	0.001	0.001	0.001	0.029	0.029	0.028	0.029	0.028
		30	0.001	0.002	0.002	0.002	0.059	0.061	0.061	0.060	0.059
4	A	15	0.002	0.002	0.002	0.002	0.048	0.046	0.051	0.048	0.046
		30	0.005	0.005	0.005	0.005	0.097	0.095	0.100	0.097	0.092
	B	15	0.002	0.003	0.004	0.003	0.053	0.058	0.053	0.055	0.052
		30	0.005	0.005	0.004	0.005	0.101	0.105	0.102	0.103	0.098
7	A	15	0.001	0.000	0.000	0.000	0.066	0.067	0.068	0.067	0.067
		30	0.000	0.000	0.001	0.000	0.124	0.129	0.122	0.125	0.125
	B	15	0.001	0.000	0.000	0.000	0.069	0.067	0.067	0.068	0.067
		30	0.001	0.001	0.001	0.001	0.146	0.146	0.145	0.146	0.145
8	A	10	0.002	0.001	0.001	0.001	0.031	0.034	0.035	0.033	0.032
		20	0.002	0.002	0.003	0.002	0.069	0.076	0.072	0.072	0.070
	B	10	0.001	0.001	0.002	0.001	0.029	0.030	0.027	0.029	0.027
		20	0.001	0.002	0.002	0.002	0.065	0.060	0.059	0.061	0.060
10	A	15	0.000	0.000	0.001	0.000	0.041	0.037	0.040	0.039	0.039
		30	0.001	0.002	0.001	0.001	0.094	0.093	0.090	0.092	0.091
	B	15	0.000	0.002	0.002	0.001	0.035	0.035	0.039	0.036	0.035
		30	0.002	0.001	0.001	0.001	0.087	0.090	0.088	0.088	0.087
12	A	10	0.001	0.000	0.002	0.001	0.059	0.060	0.062	0.060	0.059
		20	0.002	0.003	0.000	0.002	0.111	0.003	0.112	0.075	0.074
	B	10	0.002	0.004	0.003	0.003	0.064	0.071	0.065	0.067	0.064
		20	0.005	0.003	0.003	0.004	0.129	0.130	0.125	0.128	0.124
14	A	15	0.002	0.002	0.002	0.002	0.075	0.072	0.075	0.074	0.072
		30	0.002	0.003	0.002	0.002	0.144	0.148	0.148	0.147	0.144
	B	15	0.001	0.003	0.002	0.002	0.090	0.092	0.094	0.092	0.090
		30	0.002	0.002	0.001	0.002	0.179	0.178	0.179	0.179	0.177

Appendix IV: Calculations for cellulases and xylanases activity

Table 13: Calculation for Cellulase activity of Pb12(pH 6.5)

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				mole/L $_{rxn}$	µmole/L $_{sample}$	µmole/L. min
1	A	15	0.000	2.43	24.32	1.62
		30	0.000	0.00	0.00	0.00
	B	15	-0.000	-2.43	-24.32	-1.62
		30	-0.001	-4.86	-48.64	-1.62
2	A	15	0.000	0.00	0.00	0.00
		30	0.001	7.30	72.96	2.43
	B	15	0.000	2.43	24.32	1.62
		30	0.003	21.89	218.88	7.30
3	A	15	0.021	150.78	1507.84	100.52
		30	0.047	342.91	3429.12	114.30
	B	15	0.028	201.86	2018.56	134.57
		30	0.059	428.03	4280.32	142.68
4	A	15	0.046	338.05	3380.48	225.37
		30	0.092	673.66	6736.64	224.55
	B	15	0.052	376.96	3769.60	251.31
		30	0.098	715.01	7150.08	238.34
7	A	15	0.067	486.40	4864.00	324.27
		30	0.125	909.57	9095.68	303.19
	B	15	0.067	491.26	4912.64	327.51
		30	0.145	1055.49	10554.88	351.83
8	A	10	0.032	233.47	2334.72	233.47
		20	0.070	510.72	5107.20	255.36
	B	10	0.027	199.42	1994.24	199.42
		20	0.060	435.33	4353.28	217.66
10	A	15	0.039	284.54	2845.44	189.70
		30	0.091	663.94	6639.36	221.31
	B	15	0.035	255.36	2553.60	170.24
		30	0.087	634.75	6347.52	211.58
12	A	10	0.059	432.90	4328.96	432.90
		20	0.074	537.47	5374.72	268.74
	B	10	0.064	464.51	4645.12	464.51
		20	0.124	907.14	9071.36	453.57
14	A	15	0.072	525.31	5253.12	350.21
		30	0.144	1053.06	10530.56	351.02
	B	15	0.090	656.64	6566.40	437.76
		30	0.177	1291.39	12913.92	430.46

Note: slope value = 7296

Table 14: Absorbance at 540 nm for cellulase activity of Pb2 (essay in pH 5)

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
1	A	10	0.000	0.000	0.000	0.000	0.002	0.002	0.003	0.002	0.002
		20	0.000	0.000	0.000	0.000	0.005	0.005	0.004	0.005	0.005
	B	10	0.001	0.000	0.001	0.001	0.003	0.004	0.004	0.004	0.003
		20	0.001	0.000	0.002	0.001	0.005	0.006	0.004	0.005	0.004
2	A	10	0.000	0.001	0.000	0.000	0.005	0.006	0.007	0.006	0.006
		20	0.000	0.001	0.001	0.001	0.014	0.015	0.016	0.015	0.014
	B	10	0.000	0.001	0.001	0.001	0.007	0.006	0.006	0.006	0.006
		20	0.001	0.001	0.001	0.001	0.015	0.013	0.013	0.014	0.013
3	A	10	0.002	0.001	0.002	0.002	0.043	0.048	0.049	0.047	0.045
		20	0.002	0.002	0.002	0.002	0.090	0.091	0.088	0.090	0.088
	B	10	0.001	0.001	0.002	0.001	0.057	0.053	0.060	0.057	0.055
		20	0.001	0.002	0.002	0.002	0.105	0.106	0.112	0.108	0.106
4	A	10	0.003	0.003	0.002	0.003	0.081	0.078	0.084	0.081	0.078
		20	0.005	0.005	0.005	0.005	0.147	0.142	0.148	0.146	0.141
	B	10	0.002	0.003	0.004	0.003	0.095	0.094	0.095	0.095	0.092
		20	0.003	0.004	0.004	0.004	0.163	0.160	0.164	0.162	0.159
7	A	10	0.002	0.002	0.001	0.002	0.137	0.134	0.142	0.138	0.136
		20	0.002	0.001	0.001	0.001	0.223	0.227	0.228	0.226	0.225
	B	10	0.001	0.000	0.001	0.001	0.151	0.153	0.154	0.153	0.152
		20	0.001	0.001	0.001	0.001	0.270	0.272	0.280	0.274	0.273
8	A	10	0.002	0.001	0.001	0.001	0.114	0.115	0.117	0.115	0.114
		20	0.001	0.002	0.001	0.001	0.212	0.210	0.205	0.209	0.208
	B	10	0.000	0.000	0.001	0.000	0.119	0.115	0.107	0.114	0.113
		20	0.001	0.001	0.001	0.001	0.213	0.209	0.211	0.211	0.210
10	A	10	0.001	0.000	0.001	0.001	0.085	0.086	0.084	0.085	0.084
		20	0.001	0.002	0.001	0.001	0.164	0.167	0.164	0.165	0.164
	B	10	0.001	0.002	0.002	0.002	0.109	0.102	0.103	0.105	0.103
		20	0.001	0.001	0.001	0.001	0.180	0.174	0.185	0.180	0.179
12	A	10	0.002	0.001	0.001	0.001	0.162	0.154	0.163	0.160	0.158
		20	0.002	0.002	0.002	0.002	0.289	0.297	0.298	0.295	0.293
	B	10	0.001	0.003	0.002	0.002	0.185	0.183	0.187	0.185	0.183
		20	0.002	0.002	0.001	0.002	0.314	0.313	0.312	0.313	0.311
14	A	10	0.002	0.002	0.001	0.002	0.147	0.148	0.157	0.151	0.149
		20	0.003	0.002	0.002	0.002	0.262	0.261	0.260	0.261	0.259
	B	10	0.001	0.002	0.000	0.001	0.192	0.195	0.188	0.192	0.191
		20	0.002	0.002	0.001	0.002	0.323	0.328	0.321	0.324	0.322

Appendix IV: Calculations for cellulases and xylanases activity

Table 15: Calculation for cellulase activity of Pb2 (pH 5)

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/$L_{rxn\ mix}$	µmole/L_{sample}	µmole/L. min
1	A	10	0.002	17.38	173.79	17.38
		20	0.005	34.76	347.58	17.38
	B	10	0.003	22.34	223.45	22.34
		20	0.004	29.79	297.93	14.90
2	A	10	0.006	42.21	422.06	42.21
		20	0.014	106.76	1067.58	53.38
	B	10	0.006	42.21	422.06	42.21
		20	0.013	94.34	943.44	47.17
3	A	10	0.045	335.17	3351.69	335.17
		20	0.088	652.96	6529.59	326.48
	B	10	0.055	412.13	4121.34	412.13
		20	0.106	789.51	7895.09	394.75
4	A	10	0.078	583.44	5834.42	583.44
		20	0.141	1047.71	10477.13	523.86
	B	10	0.092	682.75	6827.52	682.75
		20	0.159	1181.78	11817.81	590.89
7	A	10	0.136	1012.96	10129.55	1012.96
		20	0.225	1673.36	16733.62	836.68
	B	10	0.152	1132.13	11321.26	1132.13
		20	0.273	2033.36	20333.59	1016.68
8	A	10	0.114	849.09	8490.95	849.09
		20	0.208	1546.74	15467.43	773.37
	B	10	0.113	844.13	8441.29	844.13
		20	0.210	1564.12	15641.22	782.06
10	A	10	0.084	628.13	6281.32	628.13
		20	0.164	1219.02	12190.22	609.51
	B	10	0.103	767.16	7671.65	767.16
		20	0.179	1330.75	13307.45	665.37
12	A	10	0.158	1179.30	11792.98	1179.30
		20	0.293	2179.84	21798.40	1089.92
	B	10	0.183	1363.02	13630.21	1363.02
		20	0.311	2318.38	23183.76	1159.19
14	A	10	0.149	1109.78	11097.82	1109.78
		20	0.259	1926.60	19266.01	963.30
	B	10	0.191	1420.12	14201.23	1420.12
		20	0.322	2400.80	24008.03	1200.40

Note: slope value = 7296

Table 16: Absorbance at 540 nm for xylanase activity of Pb2, pH 5

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
1	A	10	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.001	0.000
		20	0.001	0.000	0.001	0.001	0.001	0.003	0.001	0.002	0.001
	B	10	0.002	0.001	0.000	0.001	0.002	0.004	0.002	0.003	0.002
		20	0.002	0.002	0.001	0.002	0.003	0.004	0.004	0.004	0.002
2	A	10	0.004	0.005	0.004	0.004	0.015	0.016	0.015	0.015	0.011
		20	0.005	0.006	0.003	0.005	0.028	0.028	0.031	0.029	0.024
	B	10	0.006	0.005	0.005	0.005	0.017	0.017	0.018	0.017	0.012
		20	0.006	0.005	0.005	0.005	0.025	0.031	0.030	0.029	0.023
3	A	10	0.004	0.004	0.003	0.004	0.101	0.105	0.106	0.104	0.100
		20	0.004	0.005	0.004	0.004	0.173	0.171	0.178	0.174	0.170
	B	10	0.008	0.007	0.009	0.008	0.121	0.116	0.128	0.122	0.114
		20	0.007	0.008	0.007	0.007	0.182	0.184	0.185	0.184	0.176
4	A	10	0.006	0.008	0.006	0.007	0.188	0.178	0.182	0.183	0.176
		20	0.009	0.008	0.008	0.008	0.222	0.227	0.225	0.225	0.216
	B	10	0.009	0.009	0.009	0.009	0.189	0.190	0.179	0.186	0.177
		20	0.008	0.009	0.008	0.008	0.262	0.270	0.328	0.287	0.278
7	A	10	0.006	0.008	0.005	0.006	0.205	0.208	0.220	0.211	0.205
		20	0.007	0.007	0.008	0.007	0.282	0.281	0.285	0.283	0.275
	B	10	0.006	0.005	0.006	0.006	0.229	0.234	0.231	0.231	0.226
		20	0.007	0.005	0.007	0.006	0.299	0.305	0.306	0.303	0.297
8	A	10	0.000	0.001	0.001	0.001	0.106	0.104	0.113	0.108	0.107
		20	0.001	0.001	0.001	0.001	0.150	0.173	0.158	0.160	0.159
	B	10	0.005	0.006	0.006	0.006	0.150	0.165	0.168	0.161	0.155
		20	0.007	0.005	0.005	0.006	0.238	0.232	0.234	0.235	0.229
10	A	10	0.005	0.006	0.007	0.006	0.141	0.134	0.137	0.137	0.131
		20	0.004	0.003	0.007	0.005	0.209	0.206	0.210	0.208	0.204
	B	10	0.006	0.004	0.004	0.005	0.118	0.120	0.127	0.122	0.117
		20	0.005	0.005	0.005	0.005	0.188	0.190	0.197	0.192	0.187
12	A	10	0.006	0.008	0.007	0.007	0.218	0.217	0.212	0.216	0.209
		20	0.008	0.008	0.007	0.008	0.314	0.315	0.320	0.316	0.309
	B	10	0.006	0.007	0.008	0.007	0.217	0.221	0.212	0.217	0.210
		20	0.008	0.008	0.006	0.007	0.322	0.313	0.315	0.317	0.309
14	A	10	0.007	0.005	0.007	0.006	0.209	0.206	0.204	0.206	0.200
		20	0.007	0.007	0.006	0.007	0.288	0.288	0.290	0.289	0.282
	B	10	0.006	0.006	0.004	0.005	0.231	0.232	0.224	0.229	0.224
		20	0.007	0.006	0.005	0.006	0.320	0.325	0.319	0.321	0.315

Appendix IV: Calculations for cellulases and xylanases activity

Table 17: Calculation for xylanase activity of Pb2, pH 5

Day	Sample	Incubation time (min)	Corrected OD	Xylose content		
				µmole/L	µmole/L. 30min	µmole/L. min
1	A	10	0.000	2.62	26.24	2.62
		20	0.001	7.87	78.73	3.94
	B	10	0.002	13.12	131.21	13.12
		20	0.002	15.75	157.45	7.87
2	A	10	0.011	86.60	866.00	86.60
		20	0.024	191.57	1915.69	95.78
	B	10	0.012	94.47	944.72	94.47
		20	0.023	183.70	1836.96	91.85
3	A	10	0.100	789.89	7898.94	789.89
		20	0.170	1335.73	13357.35	667.87
	B	10	0.114	894.86	8948.64	894.86
		20	0.176	1388.22	13882.19	694.11
4	A	10	0.176	1385.60	13855.95	1385.60
		20	0.216	1703.13	17031.27	851.56
	B	10	0.177	1393.47	13934.68	1393.47
		20	0.278	2191.23	21912.35	1095.62
7	A	10	0.205	1611.28	16112.79	1611.28
		20	0.275	2167.62	21676.17	1083.81
	B	10	0.226	1776.61	17766.06	1776.61
		20	0.297	2338.19	23381.92	1169.10
8	A	10	0.107	842.38	8423.79	842.38
		20	0.159	1254.38	12543.84	627.19
	B	10	0.155	1222.89	12228.93	1222.89
		20	0.229	1802.85	18028.48	901.42
10	A	10	0.131	1033.95	10339.48	1033.95
		20	0.204	1603.41	16034.07	801.70
	B	10	0.117	921.11	9211.06	921.11
		20	0.187	1469.57	14695.71	734.79
12	A	10	0.209	1642.77	16427.70	1642.77
		20	0.309	2430.04	24300.40	1215.02
	B	10	0.210	1650.64	16506.43	1650.64
		20	0.309	2435.29	24352.89	1217.64
14	A	10	0.200	1574.54	15745.40	1574.54
		20	0.282	2220.10	22201.01	1110.05
	B	10	0.224	1760.86	17608.61	1760.86
		20	0.315	2482.52	24825.25	1241.26

Note: slope value = 7872.7

Table 18: Absorbance at 540 nm for cellulase activity of Tb1

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
2	A	10	0.134	0.137	0.135	0.135	0.225	0.227	0.220	0.224	0.089
		20	0.158	0.160	0.165	0.161	0.252	0.260	0.259	0.257	0.096
	B	10	0.130	0.138	0.132	0.133	0.226	0.233	0.228	0.229	0.096
		20	0.142	0.139	0.140	0.140	0.236	0.230	0.235	0.234	0.093
	C	10	0.139	0.140	0.143	0.141	0.236	0.235	0.235	0.235	0.095
		20	0.177	0.180	0.185	0.181	0.471	0.498	0.499	0.489	0.309
3	A	10	0.176	0.181	0.178	0.178	0.259	0.259	0.262	0.260	0.082
		20	0.186	0.189	0.190	0.188	0.261	0.262	0.265	0.263	0.074
	B	10	0.202	0.197	0.201	0.200	0.235	0.237	0.229	0.234	0.034
		20	0.198	0.202	0.205	0.202	0.227	0.226	0.224	0.226	0.024
	C	10	0.209	0.210	0.205	0.208	0.234	0.246	0.240	0.240	0.032
		20	0.210	0.207	0.213	0.210	0.227	0.231	0.235	0.231	0.021
6	A	10	0.001	0.001	0.002	0.001	0.005	0.004	0.005	0.005	0.003
		20	0.003	0.003	0.004	0.003	0.007	0.008	0.008	0.008	0.004
	B	10	0.018	0.015	0.017	0.017	0.011	0.026	0.023	0.025	0.008
		20	0.020	0.018	0.017	0.018	0.028	0.032	0.030	0.030	0.012
	C	10	0.069	0.074	0.075	0.073	0.088	0.087	0.083	0.086	0.013
		20	0.072	0.067	0.070	0.070	0.088	0.084	0.086	0.086	0.016
10	A	10	0.001	0.002	0.001	0.001	0.003	0.003	0.004	0.003	0.002
		20	0.001	0.001	0.002	0.001	0.005	0.005	0.005	0.005	0.004
	B	10	0.002	0.001	0.002	0.002	0.002	0.001	0.002	0.002	0.000
		20	0.001	0.002	0.001	0.001	0.003	0.002	0.001	0.002	0.001
	C	10	0.001	0.001	0.000	0.001	0.002	0.002	0.001	0.002	0.001
		20	0.001	0.001	0.001	0.001	0.004	0.002	0.005	0.004	0.003
13	A	10	0.000	0.000	0.001	0.000	0.001	0.002	0.002	0.002	0.001
		20	0.001	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.001
	B	10	0.001	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.000
		20	0.002	0.001	0.002	0.002	0.002	0.003	0.002	0.002	0.001
	C	10	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001
		20	0.002	0.000	0.001	0.001	0.000	0.000	0.002	0.001	0.000
16	A	10	0.001	0.001	0.002	0.001	0.003	0.002	0.004	0.003	0.002
		20	0.000	0.001	0.002	0.001	0.004	0.005	0.003	0.004	0.003
	B	10	0.000	0.002	0.000	0.001	0.003	0.005	0.002	0.003	0.003
		20	0.001	0.002	0.002	0.002	0.004	0.002	0.006	0.004	0.002
	C	10	0.000	0.000	0.000	0.000	0.002	0.002	0.003	0.002	0.002
		20	0.002	0.001	0.001	0.001	0.004	0.004	0.005	0.004	0.003

Appendix IV: Calculations for cellulases and xylanases activity

Table 19: Calculation for cellulase activity of Tb1

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
2	A	10	0.089	646.9	6469.1	646.9
		20	0.096	700.4	7004.2	350.2
	B	10	0.096	698.0	6979.8	698.0
		20	0.093	681.0	6809.6	340.5
	C	10	0.095	690.7	6906.9	690.7
		20	0.309	2252.0	22520.3	1126.0
3	A	10	0.082	595.8	5958.4	595.8
		20	0.074	542.3	5423.4	271.2
	B	10	0.034	245.6	2456.3	245.6
		20	0.024	175.1	1751.0	87.6
	C	10	0.032	233.5	2334.7	233.5
		20	0.021	153.2	1532.2	76.6
6	A	10 (10µl)	0.003	24.8	496.5	49.7
		20(10µl)	0.004	32.3	645.5	32.3
	B	10	0.008	62.1	620.7	62.1
		20	0.012	86.9	869.0	43.4
	C	10	0.013	99.3	993.1	99.3
		20	0.016	121.7	1216.5	60.8
10	A	10	0.002	14.9	149.0	14.9
		20	0.004	27.3	273.1	13.7
	B	10	0.000	0.0	0.0	0.0
		20	0.001	5.0	49.7	2.5
	C	10	0.001	7.4	74.5	7.4
		20	0.003	19.9	198.6	9.9
13	A	10	0.001	9.9	99.3	9.9
		20	0.001	7.4	74.5	3.7
	B	10	0.000	0.0	0.0	0.0
		20	0.001	5.0	49.7	2.5
	C	10	0.001	7.4	74.5	7.4
		20	0.000	-2.5	-24.8	-1.2
16	A	10	0.002	12.4	124.1	12.4
		20	0.003	22.3	223.4	11.2
	B	10	0.003	19.9	198.6	19.9
		20	0.002	17.4	173.8	8.7
	C	10	0.002	17.4	173.8	17.4
		20	0.003	22.3	223.4	11.2

Note: Slope value = 7448.2 except Day 2 & 3 = 7296

Table 20: Absorbance at 540 nm for xylanase activity of Tb1

Day	Sample	Incubation time (min)	Control			Average	Enzyme supernatant			Average	Corrected OD
			1	2	3		1	2	3		
2	A	30	0.175	0.177	0.179	0.177	0.181	0.175	0.176	0.177	0.000
	B	30	0.165	0.166	0.167	0.166	0.264	0.268	0.272	0.268	0.102
	C	30	0.179	0.171	0.175	0.175	0.253	0.252	0.253	0.253	0.078
3	A	30	0.222	0.230	0.225	0.226	0.398	0.415	0.401	0.405	0.179
	B	30	0.100	0.112	0.140	0.117	0.243	0.268	0.255	0.255	0.138
	C	30	0.078	0.097	0.085	0.087	0.335	0.342	0.354	0.344	0.257
6	A	30	0.016	0.022	0.017	0.018	0.046	0.038	0.084	0.056	0.038
	B	30	0.050	0.040	0.049	0.046	0.064	0.039	0.084	0.062	0.016
	C	30	0.090	0.083	0.093	0.089	0.158	0.144	0.148	0.150	0.061
10	A	30	0.007	0.002	0.002	0.004	0.029	0.028	0.035	0.031	0.027
	B	30	0.001	0.003	0.007	0.004	0.004	0.005	0.005	0.005	0.001
	C	30	0.002	0.004	0.000	0.002	0.007	0.007	0.007	0.007	0.005
13	A	30	0.004	0.006	0.004	0.005	0.025	0.023	0.025	0.024	0.020
	B	30	0.006	0.005	0.006	0.006	0.007	0.008	0.010	0.008	0.003
	C	30	0.006	0.007	0.006	0.006	0.006	0.006	0.007	0.006	0.000
16	A	30	0.008	0.007	0.007	0.007	0.009	0.008	0.008	0.008	0.001
	B	30	0.007	0.008	0.006	0.007	0.008	0.008	0.007	0.008	0.001
	C	30	0.006	0.010	0.007	0.008	0.008	0.009	0.009	0.009	0.001

Table 21: Calculation for xylanase activity of Tb1

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
2	A	30	0.000	2.7	27.0	0.9
	B	30	0.102	826.2	8262.1	275.4
	C	30	0.078	629.1	6291.1	209.7
3	A	30	0.179	1449.9	14499.2	483.3
	B	30	0.138	1117.8	11178.1	372.6
	C	30	0.257	2081.7	20817.3	693.9
6	A	30	0.038	305.1	3051.0	101.7
	B	30	0.016	129.6	1296.0	43.2
	C	30	0.061	496.8	4968.1	165.6
10	A	30	0.027	218.7	2187.0	72.9
	B	30	0.001	8.1	81.0	2.7
	C	30	0.005	40.5	405.0	13.5
13	A	30	0.020	154.8	1548.3	51.6
	B	30	0.003	21.0	209.9	7.0
	C	30	0.000	0.0	0.0	0.0
16	A	30	0.001	7.9	78.7	2.6
	B	30	0.001	5.2	52.5	1.7
	C	30	0.001	7.9	78.7	2.6

Note: slope value = 8100.1

Appendix IV: Calculations for cellulases and xylanases activity

Table 22: Absorbance at 540 nm for cellulase activity of Tb2

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
1	A	15	0.040	0.044	0.045	0.043	0.191	0.187	0.192	0.190	0.147
		30	0.109	0.114	0.115	0.113	0.323	0.320	0.316	0.320	0.207
	B	15	0.037	0.039	0.040	0.039	0.152	0.151	0.154	0.152	0.114
		30	0.089	0.094	0.093	0.092	0.283	0.287	0.284	0.285	0.193
2	A	15	0.028	0.030	0.030	0.029	0.179	0.179	0.183	0.180	0.151
		30	0.071	0.074	0.072	0.072	0.283	0.286	0.284	0.284	0.212
	B	15	0.038	0.037	0.040	0.038	0.174	0.175	0.174	0.174	0.136
		30	0.084	0.085	0.082	0.084	0.292	0.291	0.296	0.293	0.209
3	A	15	0.035	0.038	0.037	0.037	0.318	0.319	0.316	0.318	0.281
		30	0.071	0.067	0.068	0.069	0.398	0.394	0.395	0.396	0.327
	B	15	0.000	0.000	0.000	0.000	0.312	0.310	0.314	0.312	0.312
		30	0.006	0.004	0.004	0.005	0.417	0.414	0.408	0.413	0.408
4	A	15	0.050	0.055	0.055	0.053	0.340	0.342	0.339	0.340	0.287
		30	0.129	0.134	0.127	0.130	0.428	0.424	0.420	0.424	0.294
	B	15	0.050	0.046	0.045	0.047	0.322	0.316	0.323	0.320	0.273
		30	0.102	0.105	0.102	0.103	0.436	0.432	0.429	0.432	0.329
7	A	10	0.015	0.015	0.016	0.015	0.306	0.301	0.309	0.305	0.290
		20	0.053	0.051	0.057	0.054	0.445	0.453	0.446	0.448	0.394
	B	10	0.009	0.010	0.010	0.010	0.292	0.301	0.295	0.296	0.286
		20	0.043	0.044	0.042	0.043	0.393	0.409	0.396	0.399	0.356
8	A	15	0.052	0.049	0.051	0.051	0.304	0.301	0.297	0.301	0.250
		30	0.098	0.096	0.096	0.097	0.408	0.409	0.403	0.407	0.310
	B	15	0.030	0.030	0.033	0.031	0.307	0.308	0.304	0.306	0.275
		30	0.073	0.075	0.070	0.073	0.403	0.408	0.398	0.403	0.330
10	A	10	0.021	0.022	0.019	0.021	0.243	0.245	0.248	0.245	0.225
		20	0.047	0.044	0.046	0.046	0.366	0.380	0.393	0.380	0.334
	B	10	0.041	0.040	0.038	0.040	0.263	0.258	0.250	0.257	0.217
		20	0.080	0.080	0.078	0.079	0.315	0.320	0.327	0.321	0.241
12	A	15	0.040	0.038	0.037	0.038	0.319	0.325	0.320	0.321	0.283
		30	0.085	0.082	0.084	0.084	0.425	0.430	0.431	0.429	0.345
	B	15	0.046	0.051	0.050	0.049	0.293	0.290	0.295	0.293	0.244
		30	0.097	0.095	0.093	0.095	0.395	0.393	0.396	0.395	0.300
14	A	15	0.046	0.045	0.046	0.046	0.327	0.328	0.321	0.325	0.280
		30	0.097	0.096	0.097	0.097	0.420	0.418	0.415	0.418	0.321
	B	15	0.042	0.041	0.041	0.041	0.277	0.283	0.279	0.280	0.238
		30	0.082	0.087	0.082	0.084	0.358	0.360	0.362	0.360	0.276

Table 23: Calculation for Cellulase activity of Tb2

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
1	A	15	0.147	1072.51	10725.12	715.01
		30	0.207	1510.27	15102.72	503.42
	B	15	0.114	829.31	8293.12	552.87
		30	0.193	1405.70	14056.96	468.57
2	A	15	0.151	1101.70	11016.96	734.46
		30	0.212	1546.75	15467.52	515.58
	B	15	0.136	992.26	9922.56	661.50
		30	0.209	1527.30	15272.96	509.10
3	A	15	0.281	2050.18	20501.76	1366.78
		30	0.327	2385.79	23857.92	795.26
	B	15	0.312	2276.35	22763.52	1517.57
		30	0.408	2979.20	29792.00	993.07
4	A	15	0.287	2093.95	20939.52	1395.97
		30	0.294	2145.02	21450.24	715.01
	B	15	0.273	1994.24	19942.40	1329.49
		30	0.329	2402.82	24028.16	800.94
7	A	10	0.290	2115.84	21158.40	2115.84
		20	0.394	2877.06	28770.56	1438.53
	B	10	0.286	2089.09	20890.88	2089.09
		20	0.356	2599.81	25998.08	1299.90
8	A	15	0.250	1824.00	18240.00	1216.00
		30	0.310	2261.76	22617.60	753.92
	B	15	0.275	2008.83	20088.32	1339.22
		30	0.330	2410.11	24101.12	803.37
10	A	10	0.225	1639.17	16391.68	1639.17
		20	0.334	2436.86	24368.64	1218.43
	B	10	0.217	1585.66	15856.64	1585.66
		20	0.241	1760.77	17607.68	880.38
12	A	15	0.283	2064.77	20647.68	1376.51
		30	0.345	2517.12	25171.20	839.04
	B	15	0.244	1777.79	17777.92	1185.19
		30	0.300	2186.37	21863.68	728.79
14	A	15	0.280	2040.45	20404.48	1360.30
		30	0.321	2342.02	23420.16	780.67
	B	15	0.238	1738.88	17388.80	1159.25
		30	0.276	2016.13	20161.28	672.04

Note: slope value = 7448.2

Appendix IV: Calculations for cellulases and xylanases activity

Table 24: Absorbance at 540 nm for xylanase activity of Tb2

Day	Sample	Incubation time (min)	Control			Average	Enzyme supernatant			Average	Corrected OD
			1	2	3		1	2	3		
1	A	10	0.023	0.023	0.025	0.024	0.282	0.274	0.280	0.279	0.255
		20	0.024	0.021	0.025	0.023	0.489	0.493	0.516	0.499	0.476
	B	10	0.023	0.024	0.024	0.024	0.253	0.258	0.254	0.255	0.231
		20	0.027	0.027	0.026	0.027	0.380	0.385	0.387	0.384	0.357
2	A	10	0.025	0.021	0.024	0.023	0.310	0.293	0.295	0.299	0.276
		20	0.030	0.029	0.030	0.030	0.380	0.385	0.387	0.384	0.354
	B	10	0.023	0.024	0.023	0.023	0.319	0.315	0.316	0.317	0.293
		20	0.028	0.026	0.027	0.027	0.459	0.458	0.467	0.461	0.434
3	A	10	0.006	0.009	0.010	0.008	0.460	0.452	0.454	0.455	0.447
		20	0.014	0.012	0.014	0.013	0.521	0.536	0.530	0.529	0.516
	B	10	0.006	0.008	0.009	0.008	0.448	0.468	0.468	0.461	0.454
		20	0.010	0.009	0.010	0.010	0.621	0.630	0.635	0.629	0.619
4	A	10	0.007	0.010	0.009	0.009	0.458	0.442	0.448	0.449	0.441
		20	0.014	0.013	0.010	0.012	0.547	0.574	0.565	0.562	0.550
	B	10	0.007	0.009	0.007	0.008	0.481	0.480	0.494	0.485	0.477
		20	0.010	0.011	0.009	0.010	0.613	0.617	0.598	0.609	0.599
7	A	10	0.010	0.011	0.010	0.010	0.447	0.450	0.452	0.450	0.439
		20	0.015	0.013	0.013	0.014	0.586	0.570	0.585	0.580	0.567
	B	10	0.011	0.010	0.011	0.011	0.443	0.442	0.434	0.440	0.429
		20	0.011	0.011	0.013	0.012	0.572	0.583	0.585	0.580	0.568
8	A	10	0.013	0.013	0.011	0.012	0.308	0.292	0.299	0.300	0.287
		20	0.018	0.018	0.016	0.017	0.416	0.395	0.407	0.406	0.389
	B	10	0.011	0.013	0.013	0.012	0.323	0.314	0.312	0.316	0.304
		20	0.018	0.019	0.015	0.017	0.432	0.433	0.440	0.435	0.418
10	A	10	0.009	0.008	0.010	0.009	0.324	0.334	0.328	0.329	0.320
		20	0.013	0.013	0.014	0.013	0.191	0.194	0.184	0.190	0.176
	B	10	0.012	0.015	0.013	0.013	0.327	0.335	0.330	0.331	0.317
		20	0.014	0.016	0.015	0.015	0.376	0.381	0.385	0.381	0.366
12	A	10	0.012	0.013	0.011	0.012	0.290	0.311	0.315	0.305	0.293
		20	0.013	0.012	0.012	0.012	0.427	0.431	0.435	0.431	0.419
	B	10	0.012	0.014	0.015	0.014	0.292	0.296	0.303	0.297	0.283
		20	0.016	0.015	0.015	0.015	0.401	0.414	0.412	0.409	0.394
14	A	10	0.012	0.010	0.012	0.011	0.285	0.289	0.289	0.288	0.276
		20	0.017	0.015	0.014	0.015	0.365	0.356	0.362	0.361	0.346
	B	10	0.010	0.011	0.011	0.011	0.270	0.252	0.265	0.262	0.252
		20	0.015	0.017	0.014	0.015	0.362	0.365	0.378	0.368	0.353

Table 25: Calculation for xylanase activity of Tb2

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
1	A	10	0.255	2007.5	20075.4	2007.5
		20	0.476	3747.4	37474.1	1873.7
	B	10	0.231	1821.2	18212.2	1821.2
		20	0.357	2813.2	28131.8	1406.6
2	A	10	0.276	2172.9	21728.7	2172.9
		20	0.354	2789.6	27895.6	1394.8
	B	10	0.293	2309.3	23093.3	2309.3
		20	0.434	3419.4	34193.8	1709.7
3	A	10	0.447	3519.1	35191.0	3519.1
		20	0.516	4059.7	40596.9	2029.8
	B	10	0.454	3571.6	35715.8	3571.6
		20	0.619	4873.2	48732.0	2436.6
4	A	10	0.441	3469.2	34692.4	3469.2
		20	0.550	4327.4	43273.6	2163.7
	B	10	0.477	3757.9	37579.0	3757.9
		20	0.599	4718.4	47183.7	2359.2
7	A	10	0.439	3458.7	34587.4	3458.7
		20	0.567	4461.2	44612.0	2230.6
	B	10	0.429	3377.4	33773.9	3377.4
		20	0.568	4474.3	44743.2	2237.2
8	A	10	0.287	2262.1	22620.9	2262.1
		20	0.389	3059.9	30598.6	1529.9
	B	10	0.304	2393.3	23933.0	2393.3
		20	0.418	3288.2	32881.6	1644.1
10	A	10	0.320	2516.6	25166.4	2516.6
		20	0.176	1388.2	13882.2	694.1
	B	10	0.317	2498.3	24982.7	2498.3
		20	0.366	2878.8	28787.8	1439.4
12	A	10	0.293	2309.3	23093.3	2309.3
		20	0.419	3296.0	32960.4	1648.0
	B	10	0.283	2230.6	22306.0	2230.6
		20	0.394	3099.2	30992.2	1549.6
14	A	10	0.276	2175.5	21754.9	2175.5
		20	0.346	2721.3	27213.3	1360.7
	B	10	0.252	1981.3	19813.0	1981.3
		20	0.353	2779.1	27790.6	1389.5

Note: slope value = 7872.7

Appendix IV: Calculations for cellulases and xylanases activity

Table 26: Absorbance at 540 nm for cellulase activity of Tw

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
3	A	10	0.120	0.119	0.120	0.120	0.128	0.124	0.120	0.124	0.004
	B	10	0.178	0.173	0.177	0.176	0.179	0.180	0.187	0.182	0.006
	C	10	0.178	0.177	0.175	0.177	0.176	0.181	0.184	0.180	0.004
6	A	10	0.034	0.033	0.034	0.034	0.068	0.062	0.073	0.068	0.034
	B	10	0.038	0.037	0.035	0.037	0.083	0.075	0.070	0.076	0.039
	C	10	0.034	0.037	0.035	0.035	0.061	0.064	0.065	0.063	0.028
8	A	10	0.006	0.008	0.005	0.006	0.042	0.043	0.050	0.045	0.039
	B	10	0.003	0.002	0.002	0.002	0.051	0.049	0.048	0.049	0.047
	C	10	0.004	0.005	0.007	0.005	0.059	0.059	0.054	0.057	0.052
10	A	10	0.002	0.003	0.003	0.003	0.035	0.035	0.034	0.035	0.032
	B	10	0.003	0.002	0.003	0.003	0.042	0.043	0.042	0.042	0.040
	C	10	0.000	0.000	0.002	0.001	0.042	0.043	0.039	0.041	0.041
13	A	10	0.002	0.002	0.001	0.002	0.020	0.018	0.020	0.019	0.018
	B	10	0.005	0.006	0.004	0.005	0.037	0.037	0.034	0.036	0.031
	C	10	0.001	0.002	0.001	0.001	0.027	0.032	0.027	0.029	0.027
15	A	10	0.002	0.001	0.001	0.001	0.023	0.024	0.024	0.024	0.022
	B	10	0.003	0.002	0.003	0.003	0.036	0.029	0.028	0.031	0.028
	C	10	0.000	0.002	0.001	0.001	0.031	0.028	0.027	0.029	0.028

Table 27: Calculation for cellulase activity of Tw

Day	Sample	Incubation time	Corrected OD	Enzyme activity		
		(min)		µmole/L $_{rxn\ mix}$	µmole/L $_{sample}$	µmole/L. min
3	A	10	0.004	32.3	3.2	0.3
	B	10	0.006	44.7	4.5	0.4
	C	10	0.004	27.3	2.7	0.3
6	A	10	0.034	253.2	25.3	2.5
	B	10	0.039	293.0	29.3	2.9
	C	10	0.028	208.5	20.9	2.1
8	A	10	0.039	288.0	28.8	2.9
	B	10	0.047	350.1	35.0	3.5
	C	10	0.052	387.3	38.7	3.9
10	A	10	0.032	238.3	23.8	2.4
	B	10	0.040	295.4	29.5	3.0
	C	10	0.041	302.9	30.3	3.0
13	A	10	0.018	131.6	13.2	1.3
	B	10	0.031	230.9	23.1	2.3
	C	10	0.027	203.6	20.4	2.0
15	A	10	0.022	166.3	16.6	1.7
	B	10	0.028	211.0	21.1	2.1
	C	10	0.028	206.1	20.6	2.1

Note: slope value = 7448.2

Table 28: Absorbance at 540 nm for xylanase activity of Tw

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
3	A	10	0.148	0.132	0.135	0.138	0.182	0.179	0.185	0.182	0.044
	B	10	0.203	0.213	0.206	0.207	0.250	0.261	0.253	0.255	0.047
	C	10	0.152	0.161	0.155	0.156	0.237	0.239	0.232	0.236	0.080
6	A	10	0.067	0.070	0.064	0.067	0.207	0.199	0.207	0.204	0.137
	B	10	0.056	0.065	0.066	0.062	0.221	0.228	0.227	0.225	0.163
	C	10	0.037	0.036	0.039	0.037	0.192	0.196	0.209	0.199	0.162
8	A	10	0.027	0.028	0.029	0.028	0.280	0.281	0.279	0.280	0.252
	B	10	0.024	0.030	0.032	0.029	0.310	0.298	0.306	0.305	0.276
	C	10	0.032	0.030	0.028	0.030	0.285	0.289	0.290	0.288	0.258
10	A	10	0.040	0.050	0.050	0.047	0.187	0.206	0.183	0.192	0.145
	B	10	0.049	0.055	0.050	0.051	0.185	0.188	0.198	0.190	0.139
	C	10	0.036	0.042	0.036	0.038	0.200	0.210	0.188	0.199	0.161
13	A	10	0.051	0.049	0.044	0.048	0.116	0.119	0.120	0.118	0.070
	B	10	0.048	0.053	0.045	0.049	0.145	0.149	0.144	0.146	0.097
	C	10	0.034	0.035	0.030	0.033	0.123	0.131	0.124	0.126	0.093
15	A	10	0.038	0.037	0.040	0.038	0.127	0.128	0.132	0.129	0.091
	B	10	0.035	0.040	0.039	0.038	0.125	0.131	0.128	0.128	0.090
	C	10	0.032	0.035	0.032	0.033	0.135	0.125	0.125	0.128	0.095

Table 29: Calculation for xylanase activity of Tw

Day	Sample	Incubation time	Corrected OD	Enzyme activity		
		(min)		µmole/$L_{rxn\ mix}$	µmole/L_{sample}	µmole/L. min
3	A	10	0.044	343.8	34.4	3.4
	B	10	0.047	372.6	37.3	3.7
	C	10	0.080	629.8	63.0	6.3
6	A	10	0.137	1081.2	108.1	10.8
	B	10	0.163	1283.3	128.3	12.8
	C	10	0.162	1272.8	127.3	12.7
8	A	10	0.252	1983.9	198.4	19.8
	B	10	0.276	2172.9	217.3	21.7
	C	10	0.258	2031.2	203.1	20.3
10	A	10	0.145	1144.2	114.4	11.4
	B	10	0.139	1094.3	109.4	10.9
	C	10	0.161	1270.1	127.0	12.7
13	A	10	0.070	553.7	55.4	5.5
	B	10	0.097	766.3	76.6	7.7
	C	10	0.093	732.2	73.2	7.3
15	A	10	0.091	713.8	71.4	7.1
	B	10	0.090	708.5	70.9	7.1
	C	10	0.095	750.5	75.1	7.5

Note: slope value = 7872.7

Appendix IV: Calculations for cellulases and xylanases activity

Table 30: Absorbance at 540 nm for cellulase activity of SSCTw1

Day	Sample	Incubation time (min)	Control			Average	Enzyme supernatant			Average	Corrected OD
			1	2	3		1	2	3		
6	A	15	0.037	0.037	0.040	0.038	0.142	0.144	0.139	0.142	0.104
		30	0.040	0.041	0.039	0.040	0.192	0.196	0.191	0.193	0.153
	B	10	0.040	0.039	0.037	0.039	0.090	0.102	0.101	0.098	0.059
		20	0.041	0.036	0.040	0.039	0.153	0.147	0.148	0.149	0.110
	C	10	0.037	0.040	0.038	0.038	0.130	0.125	0.134	0.130	0.091
		20	0.033	0.032	0.035	0.033	0.167	0.173	0.175	0.172	0.138
10	A	10	0.025	0.023	0.025	0.024	0.045	0.041	0.044	0.043	0.019
		20	0.023	0.022	0.019	0.021	0.050	0.050	0.051	0.050	0.029
	B	10	0.024	0.025	0.027	0.025	0.035	0.039	0.039	0.038	0.012
		20	0.024	0.023	0.025	0.024	0.048	0.048	0.052	0.049	0.025
	C	10	0.027	0.029	0.026	0.027	0.047	0.052	0.051	0.050	0.023
		20	0.027	0.027	0.028	0.027	0.060	0.060	0.062	0.061	0.033
13	A	10	0.034	0.035	0.037	0.035	0.047	0.051	0.050	0.049	0.014
		20	0.042	0.040	0.043	0.042	0.066	0.065	0.069	0.067	0.025
	B	10	0.022	0.021	0.022	0.022	0.029	0.029	0.028	0.029	0.007
		20	0.020	0.023	0.024	0.022	0.038	0.035	0.040	0.038	0.015
	C	10	0.025	0.029	0.026	0.027	0.029	0.031	0.028	0.029	0.003
		20	0.031	0.029	0.030	0.030	0.040	0.035	0.038	0.038	0.008
15	A	10	0.037	0.038	0.035	0.037	0.056	0.058	0.057	0.057	0.020
		20	0.040	0.039	0.036	0.038	0.070	0.073	0.069	0.071	0.032
	B	10	0.038	0.041	0.036	0.038	0.048	0.047	0.045	0.047	0.008
		20	0.040	0.041	0.037	0.039	0.050	0.052	0.049	0.050	0.011
	C	10	0.040	0.042	0.039	0.040	0.048	0.049	0.047	0.048	0.008
		20	0.041	0.037	0.043	0.040	0.054	0.054	0.056	0.055	0.014
17	A	10	0.031	0.029	0.028	0.029	0.031	0.038	0.036	0.035	0.006
		20	0.031	0.030	0.032	0.031	0.042	0.041	0.042	0.042	0.011
	B	10	0.024	0.021	0.022	0.022	0.031	0.034	0.033	0.033	0.010
		20	0.023	0.024	0.023	0.023	0.037	0.034	0.041	0.037	0.014
	C	10	0.038	0.039	0.038	0.038	0.049	0.048	0.051	0.049	0.011
		20	0.038	0.042	0.040	0.040	0.057	0.055	0.056	0.056	0.016
20	A	10	0.034	0.033	0.035	0.034	0.039	0.040	0.039	0.039	0.005
		20	0.033	0.036	0.036	0.035	0.046	0.048	0.049	0.048	0.013
	B	10	0.038	0.033	0.037	0.036	0.043	0.042	0.043	0.043	0.007
		20	0.039	0.040	0.037	0.039	0.049	0.045	0.046	0.047	0.008
	C	10	0.033	0.035	0.035	0.034	0.035	0.035	0.036	0.035	0.001
		20	0.035	0.036	0.035	0.035	0.041	0.042	0.043	0.042	0.007
22	A	10	0.036	0.040	0.037	0.038	0.035	0.036	0.035	0.035	-0.002
		20	0.040	0.042	0.038	0.040	0.040	0.046	0.044	0.043	0.003
	B	10	0.038	0.039	0.036	0.038	0.038	0.035	0.039	0.037	-0.000
		20	0.040	0.041	0.040	0.040	0.045	0.047	0.043	0.045	0.005
	C	10	0.053	0.055	0.051	0.053	0.055	0.052	0.055	0.054	0.001
		20	0.050	0.047	0.048	0.048	0.061	0.064	0.063	0.063	0.014

Table 31: Calculation for cellulase activity of SSCTw1

Day	Sample	Incubation time (min)	Corrected OD	Enzyme activity		
				µmole/L $_{rxn\ mix}$	µmole/L $_{samp}$	µmole/L. min
6	A*	15	0.104	756.35	7563.52	504.23
		30	0.153	1116.29	11162.88	372.10
	B	10	0.059	439.44	4394.44	439.44
		20	0.110	821.78	8217.85	410.89
	C	10	0.091	680.27	6802.69	680.27
		20	0.138	1030.33	10303.34	515.17
10	A	10	0.019	141.52	1415.16	141.52
		20	0.029	216.00	2159.98	108.00
	B	10	0.012	91.86	918.61	91.86
		20	0.025	188.69	1886.88	94.34
	C	10	0.023	168.83	1688.26	168.83
		20	0.033	248.27	2482.73	124.14
13	A	10	0.014	104.27	1042.75	104.27
		20	0.025	186.21	1862.05	93.10
	B	10	0.007	52.14	521.37	52.14
		20	0.015	114.21	1142.06	57.10
	C	10	0.003	19.86	198.62	19.86
		20	0.008	57.10	571.03	28.55
15	A	10	0.020	151.45	1514.47	151.45
		20	0.032	240.83	2408.25	120.41
	B	10	0.008	62.07	620.68	62.07
		20	0.011	81.93	819.30	40.97
	C	10	0.008	57.10	571.03	57.10
		20	0.014	106.76	1067.58	53.38
17	A	10	0.006	42.21	422.06	42.21
		20	0.011	79.45	794.47	39.72
	B	10	0.010	76.96	769.65	76.96
		20	0.014	104.27	1042.75	52.14
	C	10	0.011	81.93	819.30	81.93
		20	0.016	119.17	1191.71	59.59
20	A	10	0.005	39.72	397.24	39.72
		20	0.013	94.34	943.44	47.17
	B	10	0.007	49.65	496.55	49.65
		20	0.008	59.59	595.86	29.79
	C	10	0.001	7.45	74.48	7.45
		20	0.007	49.65	496.55	24.83
22	A	10	-0.002	-17.38	-173.79	-17.38
		20	0.003	24.83	248.27	12.41
	B	10	-0.000	-2.48	-24.83	-2.48
		20	0.005	34.76	347.58	17.38
	C	10	0.001	7.45	74.48	7.45
		20	0.014	106.76	1067.58	53.38

Note: slope value = 7448.2 except for * = 7296

Appendix IV: Calculations for cellulases and xylanases activity

Table 32: Absorbance at 540 nm for xylanase activity of SSCTw1

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
6	A	15	0.051	0.050	0.053	0.051	1.049	1.119	1.099	1.089	1.038
		30	0.051	0.048	0.051	0.050	1.322	1.346	1.404	1.357	1.307
	B	15	0.060	0.061	0.056	0.059	1.121	1.121	1.125	1.122	1.063
		30	0.050	0.053	0.059	0.054	1.230	1.300	1.230	1.253	1.199
	C	15	0.061	0.062	0.059	0.061	1.196	1.210	1.220	1.209	1.148
		30	0.056	0.057	0.057	0.057	1.342	1.404	1.400	1.382	1.325
10	A	15	0.049	0.050	0.047	0.049	0.964	0.957	0.978	0.966	0.918
		30	0.042	0.040	0.043	0.042	1.231	1.229	1.182	1.214	1.172
	B	15	0.038	0.042	0.039	0.040	0.944	0.953	0.955	0.951	0.911
		30	0.044	0.042	0.040	0.042	1.179	1.222	1.170	1.190	1.148
	C	15	0.047	0.050	0.047	0.048	0.971	0.941	0.965	0.959	0.911
		30	0.047	0.042	0.047	0.045	1.173	1.201	1.238	1.204	1.159
13	A	10	0.028	0.025	0.024	0.026	0.412	0.407	0.415	0.411	0.386
		20	0.023	0.026	0.027	0.025	0.558	0.570	0.568	0.565	0.540
	B	10	0.018	0.017	0.018	0.018	0.410	0.409	0.401	0.407	0.389
		20	0.014	0.018	0.017	0.016	0.541	0.549	0.545	0.545	0.529
	C	10	0.022	0.021	0.020	0.021	0.466	0.471	0.467	0.468	0.447
		20	0.018	0.020	0.018	0.019	0.609	0.610	0.615	0.611	0.593
15	A	10	0.025	0.024	0.022	0.024	0.443	0.442	0.449	0.445	0.421
		20	0.025	0.025	0.023	0.024	0.594	0.600	0.583	0.592	0.568
	B	10	0.027	0.027	0.026	0.027	0.369	0.372	0.378	0.373	0.346
		20	0.027	0.028	0.026	0.027	0.492	0.501	0.495	0.496	0.469
	C	10	0.020	0.019	0.023	0.021	0.431	0.440	0.433	0.435	0.414
		20	0.024	0.023	0.021	0.023	0.526	0.519	0.509	0.518	0.495
17	A	10	0.005	0.005	0.006	0.005	0.234	0.249	0.239	0.241	0.235
		20	0.011	0.007	0.007	0.008	0.345	0.347	0.350	0.347	0.339
	B	10	0.006	0.009	0.007	0.007	0.238	0.242	0.249	0.243	0.236
		20	0.007	0.008	0.008	0.008	0.358	0.365	0.362	0.362	0.354
	C	10	0.005	0.005	0.005	0.005	0.204	0.201	0.207	0.204	0.199
		20	0.006	0.005	0.005	0.005	0.299	0.302	0.309	0.303	0.298
20	A	10	0.007	0.008	0.010	0.008	0.219	0.216	0.222	0.219	0.211
		20	0.008	0.010	0.008	0.009	0.304	0.305	0.299	0.303	0.294
	B	10	0.010	0.011	0.011	0.011	0.215	0.221	0.216	0.217	0.207
		20	0.010	0.009	0.010	0.010	0.339	0.320	0.325	0.328	0.318
	C	10	0.011	0.010	0.008	0.010	0.204	0.204	0.194	0.201	0.191
		20	0.010	0.012	0.010	0.011	0.275	0.282	0.284	0.280	0.270
22	A	10	0.013	0.014	0.013	0.013	0.143	0.146	0.148	0.146	0.132
		20	0.011	0.010	0.014	0.012	0.217	0.220	0.215	0.217	0.206
	B	10	0.013	0.016	0.014	0.014	0.180	0.191	0.194	0.188	0.174
		20	0.015	0.012	0.014	0.014	0.254	0.269	0.260	0.261	0.247
	C	10	0.013	0.014	0.017	0.015	0.164	0.162	0.175	0.167	0.152
		20	0.014	0.013	0.016	0.014	0.248	0.249	0.239	0.245	0.231

Table 33: Calculation for xylanase activity of SSCTw1

Note: slope value = 7872.7 except for * = 8100.1

Day	Sample	Incubation time (min)	Sample volume (µl)	Corrected OD	Xylose content		
					µmole/L	µmole/L. 30min	µmole/L. min
6*	A	15	20	1.038	8405.2	84052.0	5603.5
		30	20	1.307	10589.5	105895.3	3529.8
	B	15	20	1.063	8613.1	86131.1	5742.1
		30	20	1.199	9714.7	97147.2	3238.2
	C	15	20	1.148	9298.9	92989.1	6199.3
		30	20	1.325	10735.3	107353.3	3578.4
10*	A	15	20	0.918	7433.2	74331.9	4955.5
		30	20	1.172	9496.0	94960.2	3165.3
	B	15	20	0.911	7379.2	73791.9	4919.5
		30	20	1.148	9301.6	93016.1	3100.5
	C	15	20	0.911	7379.2	73791.9	4919.5
		30	20	1.159	9385.3	93853.2	3128.4
13	A	10	10	0.386	3036.2	60724.8	6072.5
		20	10	0.540	4251.3	85025.2	4251.3
	B	10	10	0.389	3062.5	61249.6	6125.0
		20	10	0.529	4162.0	83240.7	4162.0
	C	10	10	0.447	3519.1	70381.9	7038.2
		20	10	0.593	4665.9	93317.7	4665.9
15	A	10	10	0.421	3314.4	66288.1	6628.8
		20	10	0.568	4471.7	89433.9	4471.7
	B	10	10	0.346	2726.6	54531.6	5453.2
		20	10	0.469	3692.3	73845.9	3692.3
	C	10	10	0.414	3259.3	65186.0	6518.6
		20	10	0.495	3899.6	77992.2	3899.6
17	A	10	5	0.235	1852.7	74108.3	7410.8
		20	5	0.339	2668.8	106753.8	5337.7
	B	10	5	0.236	1855.3	74213.3	7421.3
		20	5	0.354	2786.9	111477.4	5573.9
	C	10	5	0.199	1566.7	62666.7	6266.7
		20	5	0.298	2346.1	93842.6	4692.1
20	A	10	5	0.211	1658.5	66340.6	6634.1
		20	5	0.294	2314.6	92583.0	4629.1
	B	10	5	0.207	1627.0	65081.0	6508.1
		20	5	0.318	2506.1	100245.7	5012.3
	C	10	5	0.191	1503.7	60147.4	6014.7
		20	5	0.270	2123.0	84920.2	4246.0
22	A	10	5	0.132	1041.8	41672.8	4167.3
		20	5	0.206	1619.2	64766.1	3238.3
	B	10	5	0.174	1369.8	54794.0	5479.4
		20	5	0.247	1947.2	77887.2	3894.4
	C	10	5	0.152	1199.3	47971.0	4797.1
		20	5	0.231	1818.6	72743.7	3637.2

Appendix IV: Calculations for cellulases and xylanases activity

Table 34: Absorbance at 540 nm for cellulase activity of SSCTw2 (Repeated experiment)

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
3	1	10	0.145	0.141	0.140	0.142	0.336	0.369	0.389	0.365	0.223
		20	0.158	0.155	0.153	0.155	0.428	0.479	0.465	0.457	0.302
	2	10	0.227	0.209	0.208	0.215	0.380	0.375	0.379	0.378	0.163
		20	0.208	0.212	0.215	0.212	0.411	0.428	0.430	0.423	0.211
5	1	10	0.081	0.076	0.080	0.079	0.299	0.306	0.293	0.299	0.220
		20	0.093	0.090	0.093	0.092	0.404	0.422	0.410	0.412	0.320
7	1	10	0.087	0.090	0.088	0.088	0.303	0.313	0.303	0.306	0.218
		20	0.093	0.090	0.099	0.094	0.416	0.397	0.410	0.408	0.314
9	1	10	0.091	0.095	0.094	0.093	0.329	0.327	0.320	0.325	0.232
		20	0.102	0.105	0.100	0.102	0.419	0.415	0.422	0.419	0.316
11	1	10	0.101	0.104	0.093	0.099	0.346	0.334	0.339	0.340	0.240
		20	0.102	0.100	0.107	0.103	0.413	0.424	0.420	0.419	0.316

Table 35: Calculation for cellulase activity of SSCTw2 (Repeated experiment)

Day	Sample	Incubation time	Corrected OD	Enzyme activity		
		(min)		µmole/$L_{rxn\ mix}$	µmole/L_{sample}	µmole/L. min
3	1	10	0.223	1658.5	16584.7	1658.5
		20	0.302	2249.4	22493.6	1124.7
	2	10	0.163	1216.5	12165.4	1216.5
		20	0.211	1574.1	15740.5	787.0
5	1	10	0.220	1641.1	16410.9	1641.1
		20	0.320	2383.4	23834.2	1191.7
7	1	10	0.218	1623.7	16237.1	1623.7
		20	0.314	2336.3	23362.5	1168.1
9	1	10	0.232	1728.0	17279.8	1728.0
		20	0.316	2356.1	23561.1	1178.1
11	1	10	0.240	1790.1	17900.5	1790.1
		20	0.316	2353.6	23536.3	1176.8

Note: slope value = 7448.2

Table 36: Absorbance at 540 nm for xylanase activity of SSCTw2 (Repeated experiment)

Day	Sample	Incubation time	Control			Average	Enzyme supernatant			Average	Corrected OD
		(min)	1	2	3		1	2	3		
3	1	10	0.060	0.061	0.066	0.062	0.176	0.177	0.185	0.179	0.117
		20	0.070	0.075	0.073	0.073	0.247	0.241	0.245	0.244	0.172
	2	10	0.077	0.079	0.080	0.079	0.205	0.201	0.205	0.204	0.125
		20	0.088	0.085	0.086	0.086	0.261	0.251	0.260	0.257	0.171
5	1	10	0.037	0.038	0.035	0.037	0.257	0.249	0.251	0.252	0.216
		20	0.039	0.038	0.040	0.039	0.385	0.378	0.390	0.384	0.345
7	1	10	0.049	0.050	0.052	0.050	0.282	0.278	0.276	0.279	0.228
		20	0.053	0.050	0.051	0.051	0.408	0.410	0.392	0.403	0.352
9	1	10	0.034	0.040	0.039	0.038	0.232	0.247	0.236	0.238	0.201
		20	0.042	0.040	0.046	0.043	0.359	0.358	0.347	0.355	0.312
11	1	10	0.038	0.041	0.042	0.040	0.244	0.246	0.239	0.243	0.203
		20	0.051	0.049	0.045	0.048	0.379	0.363	0.360	0.367	0.319

Table 37: Calculation for xylanase activity of SSCTw2 (Repeated experiment)

Day	Sample	Incubation time	Corrected OD	Enzyme activity		
		(min)		µmole/$L_{rxn\ mix}$	µmole/L_{sample}	µmole/L. min
3	1	10	0.117	921.1	18422.1	1842.2
		20	0.172	1351.5	27029.6	1351.5
	2	10	0.125	984.1	19681.8	1968.2
		20	0.171	1346.2	26924.6	1346.2
5	1	10	0.216	1697.9	33957.6	3395.8
		20	0.345	2718.7	54374.1	2718.7
7	1	10	0.228	1797.6	35952.0	3595.2
		20	0.352	2771.2	55423.8	2771.2
9	1	10	0.201	1579.8	31595.8	3159.6
		20	0.312	2456.3	49125.6	2456.3
11	1	10	0.203	1595.5	31910.7	3191.1
		20	0.319	2511.4	50227.8	2511.4

Note: slope value= 7872.7

Applendix V: Calculations for laccase activity

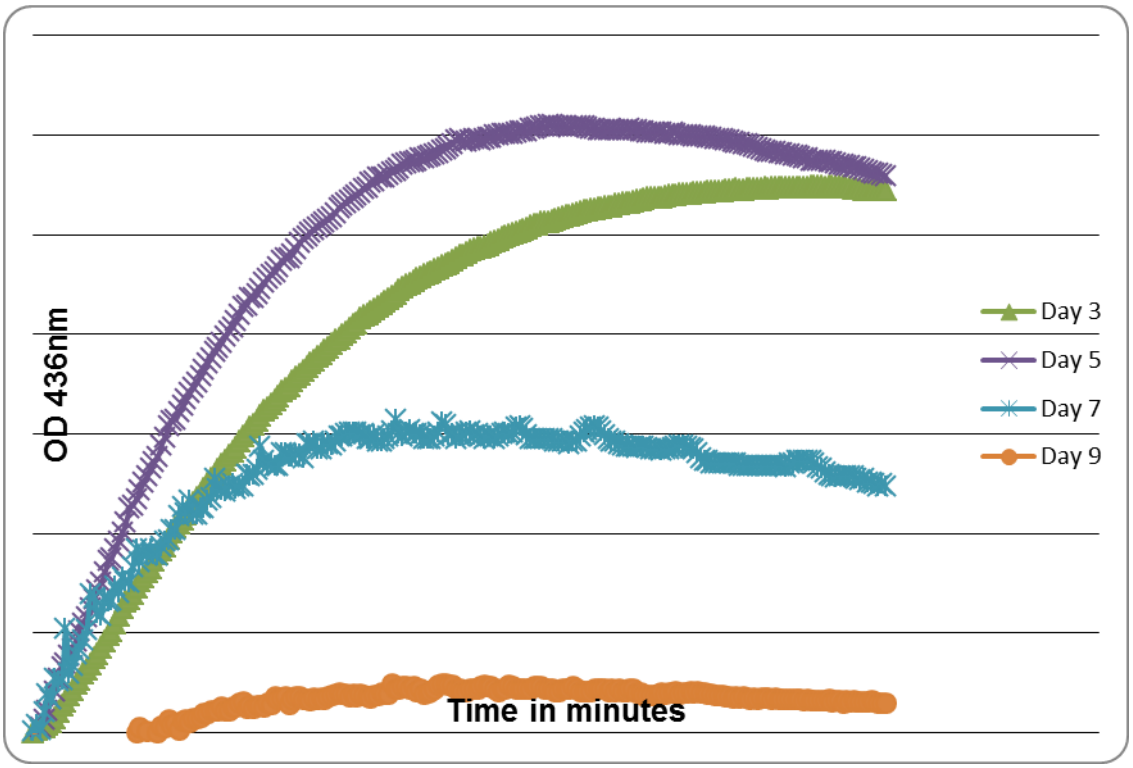

Figure 24: Change in absorbance at 436 nm for laccase assay of wheat bran SSC of *T. hirsuta* at different cultivation day

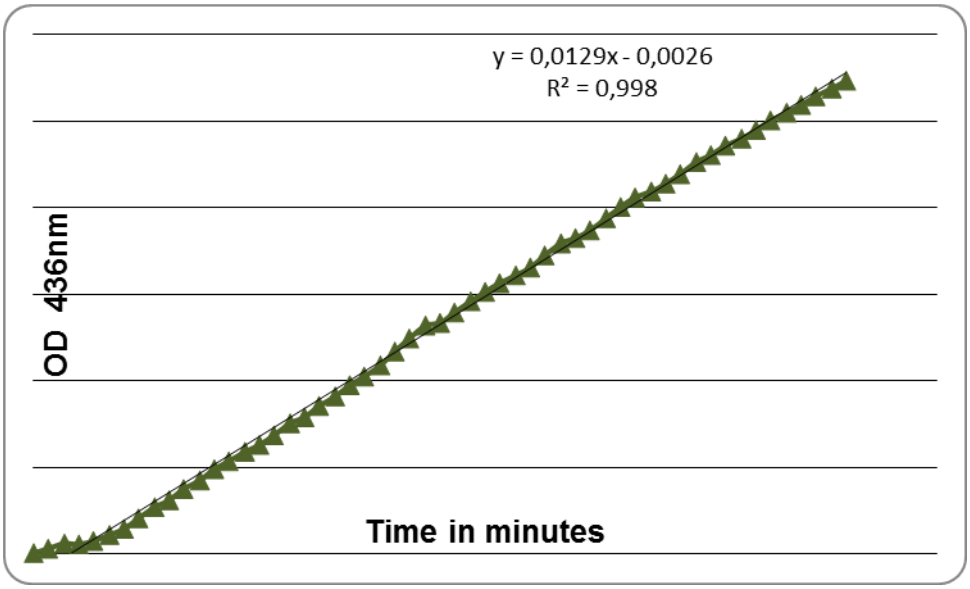

Figure 25: Change in absorbance at 436nm of day 3 sample of wheat bran SSC of *T. hirsuta*

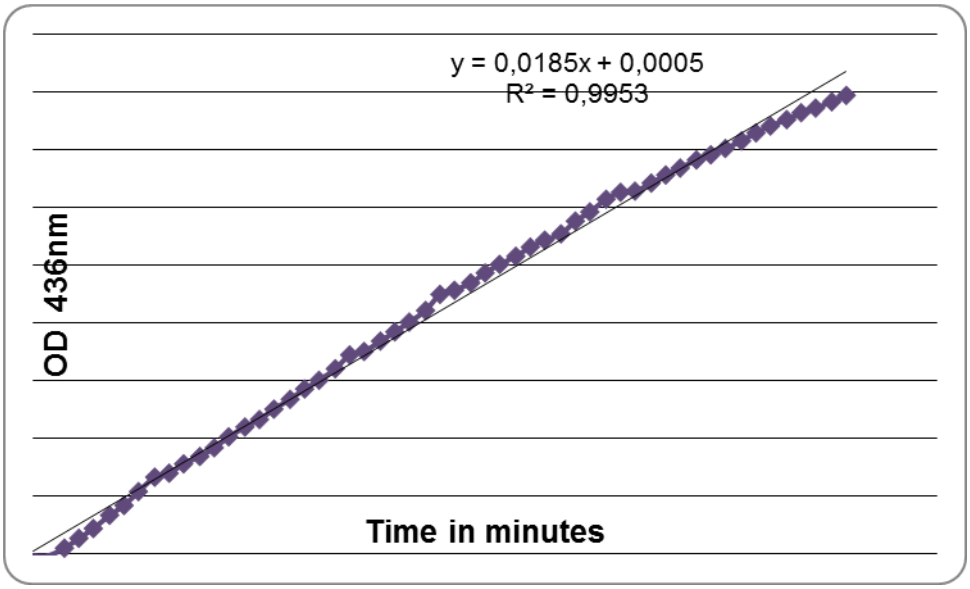

Figure 26: Change in absorbance at 436nm of day 5 sample of wheat bran SSC of *T. hirsuta*

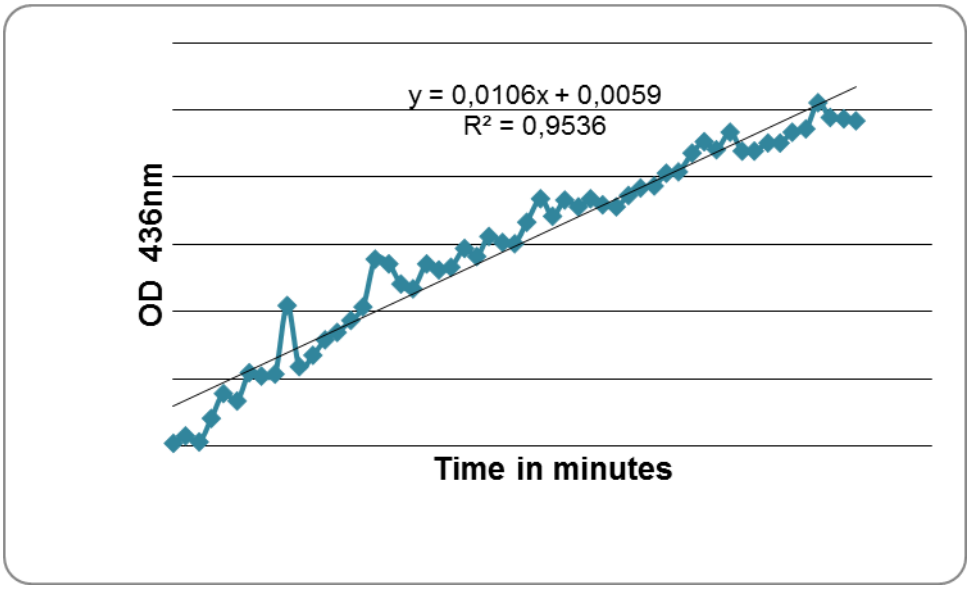

Figure 27: Change in absorbance at 436nm of day 7 sample of wheat bran SSC of *T. hirsuta*

Appendix V: Calculations for laccase activity

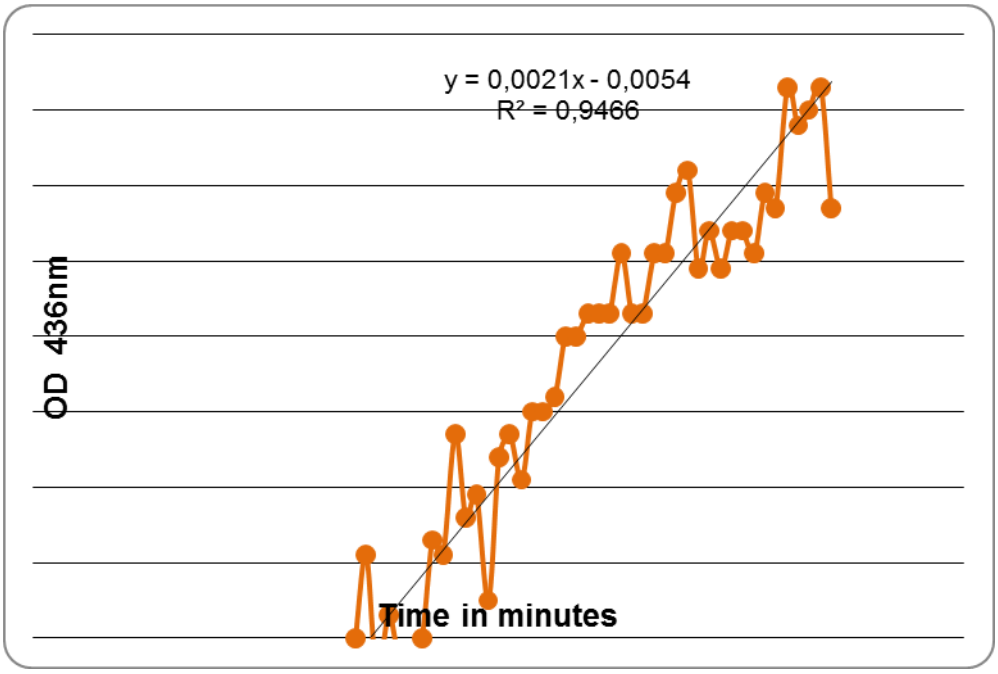

Figure 28: Change in absorbance at 436nm of day 9 sample of wheat bran SSC of *T. hirsuta*

Table 38: Calculation for laccase activity of SSCTw2

Sample	ε(M cm^{-1})	Slope (min^{-1})	Sample volume(ul)	Concentration factor	Laccase activity	
					µM per min	µM/L.min
Day 3	29300	0.012	50	17.09	0.41	479.2
Day 5	29300	0.018	50	11.36	0.61	1081.2
Day 7	29300	0.010	50	14.28	0.34	477.8
Day 9	29300	0.002	50	9.61	0.07	142.0
Day 12	29300	0	50	9.71	0.00	0.0

Calculation for laccase activity

According to Beer-Lambert law:

$A = \varepsilon cl$

Where A = Absorbance

ε = Molar absorption coefficient (M^{-1} cm^{-1})

c = Concentration (M)

l = path length (cm)

$$c = \frac{A}{\varepsilon l}$$

Given the change in absorbance per time

$$\frac{\Delta c}{\Delta t} = \frac{\Delta A}{\varepsilon l \, \Delta t}$$

Thus,

$$c = \frac{\Delta A}{\varepsilon \, \Delta t}$$

Where c = Enzyme activity (M/L.min)

ΔA = Increase in absorbance at 436 nm

ε = Molar absorption coefficient = 29300 M^{-1} cm^{-1}

Δ t = Reaction time in minute

Δ A/ Δ t = slope from graph

Thus,

$$c\ (\mu M/L.min) = \frac{(10^6 \text{ x slope})}{29300}$$

Table 39: Specific laccase activity of SSCTw2

Sample	Laccase activity (U/L)	Total protein (mg/L)	Specific laccase activity (U/mg)
Day 3	479.2	463.6	1.0
Day 5	1081.2	592.0	1.8
Day 7	477.8	606.0	0.8
Day 9	142.0	580.2	0.2
Day 12	0.0	583.0	0.0

$$\text{Specific laccase activity (U/mg)} = \frac{\text{Laccase activity (U/L)}}{\text{Total protein (mg/L)}}$$

Appendix VI: Calculation for Total protein content

Table 40: Absorbance at 595 nm of BSA standard for Bio-rad protein assay

BSA concentration (mg/l)	50	100	150	200	250	300	350	400	450
OD_{595}	0.21	0.379	0.533	0.701	0.824	0.968	1.073	1.174	1.256

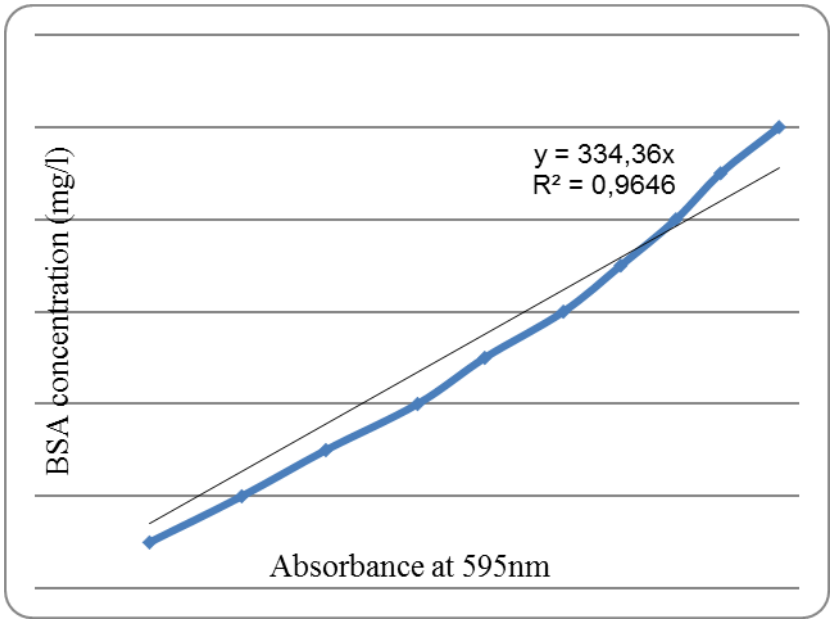

Figure 29: Standard curve for protein analysis using BSA as standard

Table 41: Total protein content of Pb1

Day	Sample	Absorbance at 595 nm			Average	Protein content (mg/L)
2	A	0.018	0.017	0.016	0.017	5.68
	B	0.011	0.007	0.012	0.010	3.34
	C	0.007	0.012	0.006	0.008	2.79
3	A	0.028	0.036	0.033	0.032	10.81
	B	0.022	0.014	0.027	0.021	7.02
	C	0.054	0.057	0.053	0.055	18.28
6	A	0.115	0.102	0.111	0.109	36.55
	B	0.065	0.064	0.059	0.063	20.95
	C	0.073	0.071	0.098	0.081	26.97
10	A	0.202	0.210	0.202	0.205	68.42
	B	0.107	0.112	0.111	0.110	36.77
	C	0.102	0.124	0.128	0.118	39.45
13	A	0.148	0.151	0.157	0.152	50.81
	B	0.112	0.126	0.131	0.123	41.12
	C	0.134	0.129	0.127	0.130	43.46
16	A	0.104	0.116	0.097	0.106	35.32
	B	0.141	0.142	0.146	0.143	47.80
	C	-	0.070	0.076	0.073	24.40

Table 42: Total protein content of Pb2

Day	Sample	Absorbance at 595 nm		Average	Protein content (mg/L)
1	A	0.103	0.092	0.098	32.59
	B	0.104	0.101	0.103	34.27
2	A	0.109	0.093	0.101	33.76
	B	0.108	0.105	0.107	35.60
3	A	0.148	0.133	0.141	46.97
	B	0.145	0.138	0.142	47.30
4	A	0.141	0.150	0.146	48.64
	B	0.147	0.161	0.154	51.48
7	A	0.103	0.118	0.111	36.94
	B	0.117	0.101	0.109	36.44
8	A	0.109	0.093	0.101	33.76
	B	0.108	0.105	0.107	35.60
10	A	0.133	0.140	0.137	45.63
	B	0.135	0.129	0.132	44.13
12	A	0.141	0.150	0.146	48.64
	B	0.147	0.161	0.154	51.48
14	A	0.201	0.193	0.197	65.86
	B	0.202	0.210	0.206	68.87

Appendix VI: Calculations for total protein content

Table 43: Total protein content of Tb1

Day	Sample	Absorbance at 595 nm		Average	Protein content (mg/L)
2	A	0.014	0.013	0.014	4.51
	B	0.019	0.017	0.018	6.02
	C	0.018	0.013	0.016	5.18
3	A	0.015	0.023	0.019	6.35
	B	0.015	0.014	0.015	4.85
	C	0.015	0.015	0.015	5.01
6	A	0.038	0.025	0.032	10.53
	B	0.022	0.017	0.020	6.52
	C	0.027	0.026	0.027	8.86
10	A	0.090	0.119	0.105	34.93
	B	0.122	0.115	0.119	39.61
	C	0.137	0.130	0.134	44.63
13	A	0.122	0.124	0.123	41.12
	B	0.114	0.109	0.112	37.27
	C	0.121	0.123	0.122	40.78
16	A	0.081	0.076	0.079	26.24
	B	0.031	0.032	0.032	10.53
	C	0.083	0.069	0.076	25.41

Table 44: Total protein content of Tb2

Day	Sample	Absorbance at 595 nm			Average	Protein content (mg/L)
1	A	0.458	0.422	0.423	0.434	145.20
	B	0.392	0.435	0.392	0.406	135.84
2	A	0.345	0.326	0.302	0.324	108.42
	B	0.302	0.300	0.316	0.306	102.30
3	A	0.320	0.350	0.358	0.343	114.55
	B	0.367	0.369	0.360	0.365	122.13
4	A	0.315	0.288	0.266	0.290	96.84
	B	0.299	-	0.284	0.292	97.45
7	A	0.342	0.351	0.330	0.341	114.00
	B	0.254	0.246	0.242	0.247	82.68
8	A	0.192	-	0.203	0.198	66.02
	B	0.169	0.138	0.155	0.154	51.48
10	A	0.321	0.319	0.313	0.318	106.20
	B	0.301	0.273	0.293	0.289	96.61
12	A	-	0.179	0.227	0.203	67.86
	B	0.186	0.192	-	0.189	63.18
14	A	0.228	0.203	-	0.216	72.04
	B	-	0.174	0.182	0.178	59.51

Table 45: Total protein content of Tw

Day	Sample	Absorbance at 595 nm			Average	Protein content (mg/L)
3	A	0.051	0.054	0.057	0.054	18.05
	B	0.061	0.071	0.058	0.063	21.17
	C	0.071	0.081	0.072	0.075	24.96
6	A	0.172	0.179	0.182	0.178	59.39
	B	0.157	0.157	0.144	0.153	51.04
	C	0.190	0.192	0.178	0.187	62.40
8	A	0.267	0.274	0.285	0.275	92.04
	B	0.250	0.287	0.286	0.274	91.71
	C	0.245	0.235	0.248	0.243	81.12
10	A	0.211	0.207	0.212	0.210	70.20
	B	0.196	0.202	0.198	0.199	66.41
	C	0.192	0.207	0.224	0.208	69.42
13	A	0.194	0.231	0.208	0.211	70.54
	B	0.198	0.221	0.240	0.220	73.43
	C	0.194	0.206	0.206	0.202	67.53
15	A	0.284	0.285	0.292	0.287	95.94
	B	0.257	0.262	0.272	0.264	88.14
	C	0.273	0.278	0.292	0.281	93.94

Table 46: Total protein content of SSCTw1

Day	Sample	Absorbance at 595 nm		Average	Protein content (mg/L)
6	A	0.986	1.011	0.986	338.08
	B	0.871	0.895	0.871	299.29
	C	1.078	1.157	1.078	386.90
10	A	0.967	0.996	0.967	333.06
	B	0.997	0.993	0.997	331.96
	C	0.991	0.983	0.991	328.62
13	A	1.046	1.048	1.046	350.35
	B	0.921	0.907	0.921	303.21
	C	0.988	0.959	0.988	320.59
15	A	0.910	0.914	0.910	305.55
	B	0.859	0.864	0.859	288.84
	C	0.894	0.880	0.894	294.18
17	A	0.883	0.904	0.883	302.21
	B	0.811	0.816	0.811	272.79
	C	0.754	0.761	0.754	254.40
20	A	0.891	0.904	0.891	302.21
	B	0.846	0.848	0.846	283.49
	C	0.853	0.849	0.853	283.82
22	A	0.808	0.832	0.808	278.14
	B	0.835	0.827	0.835	276.47
	C	0.838	0.810	0.838	270.78

Appendix VI: Calculations for total protein content

Table 47: Total protein content of SSCTw2

Day	Absorbance at 595 nm			Average	Protein content (mg/L)
3	1.452	1.332	1.376	1.387	463.56
5	1.800	-	1.742	1.771	592.05
7	1.798	1.745	1.895	1.813	605.97
9	1.790	1.736	1.681	1.736	580.23
12	1.775	1.650	1.807	1.744	583.02